Loss of heterozygosity on chromosome 17 and p53 mutation in primary human breast cancer.

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Declaration

I declare that

a) this thesis was composed by myself, and

b) the work is my own, except where stated.

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Abstract

Tumorigenesis is a multistep process involving the alteration of genes involved in the regulation of growth. Alteration of both positively acting oncogenes and negatively acting tumour suppressor genes has been identified in breast tumours. The inactivation of tumour suppressor genes often arises through a loss of genetic material which can be detected in tumours as a reduction to homozygosity at loci that are normally heterozygous. A high rate of loss of heterozygosity (LOH) at a specific chromosomal locus is indicative of tumour suppressor gene inactivation and has been used to locate tumour suppressor genes involved in a number of malignancies. A high frequency of LOH (61%) has been detected in a series of breast tumours at a site at 17p13.3 suggesting the presence of a tumour suppressor gene involved in breast cancer on chromosome 17p (Mackay *et al.*, 1988a).

Following on from this work the incidence of LOH in primary breast tumours was determined at other loci on chromosome 17p and served a two fold purpose. First, the pattern of LOH at different loci on chromosome 17 in individual breast tumours was determined in order to define a shortest region of overlap (SRO). Identification of such a region, commonly lost in all tumours showing LOH, could be used to pinpoint the position of the putative tumour suppressor gene. Secondly, as two of the probes used to investigate LOH detect loci close to the known tumour suppressor gene p53, a potential role for the p53 gene in breast cancer development could be investigated.

Two tumour samples showed LOH at the p53 locus and not at the more distal 17p13.3 site, suggesting the involvement of the p53 gene in breast tumorigenesis. Furthermore LOH at the p53 gene locus was detected in 49% of primary breast tumour samples. Exons 5-9 of the p53 gene, which contain known mutation hotspots, were examined for mutation in 78 primary breast tumours using the HOT (amplification and mismatch detection) technique. Twenty four (31%) of the breast tumours were found to possess a somatically acquired p53 mutation, mostly single base substitutions resulting in missense mutations. In tumours with data on both LOH and p53 mutation, the majority of p53 mutations were found to be accompanied by LOH, indicating the importance of the removal of the tumour suppressive function of the gene. The types and positions of p53 mutation occurring in breast tumour samples in this and other studies were analysed. Mutations occurred at a number of

sites throughout the gene although no specific hotspot was identified. A preponderance of mutations at guanine residues, suggesting exogenous factors, was also observed. p53 mutation was found to be associated with a low level of oestrogen receptor protein suggesting an association with aggressive tumour behaviour but not with patient age, menopausal status, family history, tumour size or lymph node involvement. LOH at the p53 gene locus was associated with no specific clinical/pathological parameter.

Contents

Chapter 1
Introduction

1.1 Breast Cancer.

1.1.1 Structure and development of the human breast.

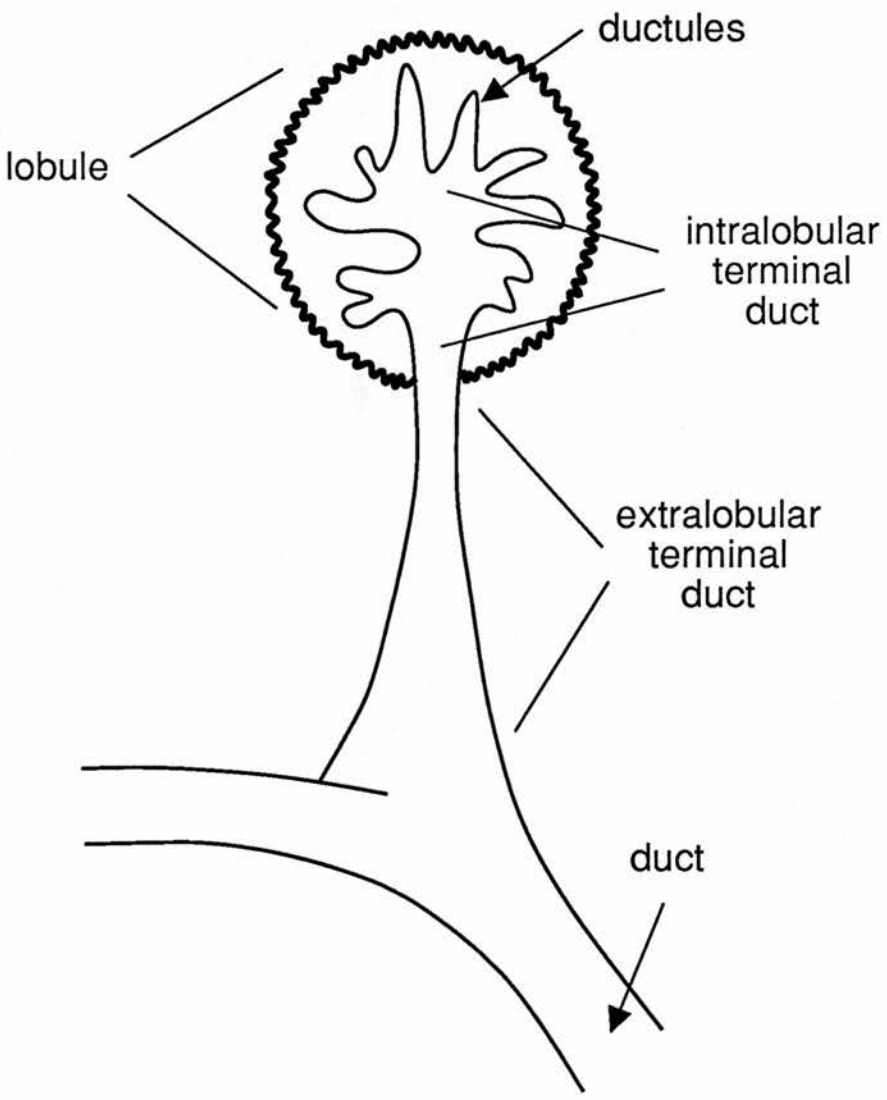
The functional component of the adult female breast comprises 15 - 20 milk producing lobular units connected to the nipple complex by a system of branching ducts. These functional units are surrounded by varying amounts of fat and connective tissue which make up the bulk of the breast. During lactation the milk produced by the lobular units drains into the duct system and is passed out of the body through the nipple (Page and Anderson 1988). The breast duct system is arranged in a segmental roughly radial pattern from the nipple and each branch consists of three parts: the ductules (DTL), which are the terminal blind endings of the duct system; and the intralobular and extralobular sections of the terminal duct (ITD and ETD), which are defined by the specialised stroma of the lobular unit surrounding the ductules. This structure is known as the terminal ductal lobular unit (TDLU) and is considered to be the basic functional unit of the breast (Figure 1.1) (Wellings *et al.*, 1975).

Major changes in the development of the breast occur during puberty. Small bundles of duct give rise to alveolar buds which when clustered form the DTL and ITD or type 1 lobule. An increase in the number of alveolar buds and the volume of the lobule results in the progressive transition of these lobular units to types 2, 3 and 4. The type 4 lobule is the most developed lobule type and is present only during pregnancy, when maximal differentiation of the breast occurs. In the absence of child bearing the lobules decrease in both size and number and only type 1 and 2 lobules are observed (Russo *et al.*, 1990).

1.1.2 Origin of human breast cancer.

Breast cancer is a heterogeneous disease and a wide variety of benign and malignant lesions of the breast have been identified. Depending upon classification there are between twenty and thirty types of breast carcinoma (Gallager 1984). Histologically, malignant breast tumours can be classed into numerous subgroups including lobular, tubular, medullary, mucinous, cribriform and papillary types although most tumours are classed as either no special type (NST) (Page and Anderson 1988) or not otherwise specified (NOS) (Fisher *et al.*, 1984). The majority of the

Figure 1.1



Normal terminal ductal lobular unit (TDLU).

malignant carcinomas are thought to arise from epithelial cells in the TDLU and are thus classified as 'ductal'. The lobular type carcinoma is thought to arise from the more differentiated type 2 lobules and type 3 and type 4 lobules have been proposed as the site of origin for benign lesions occurring in the breast (Russo *et al.*, 1990).

Breast carcinomas can be further grouped into invasive (or infiltrative) and non-invasive types depending on the presence or absence of tumour growth into the surrounding tissue. Non-invasive carcinomas are classed as ductal carcinoma in situ (DCIS) (or intraductal carcinoma) and lobular carcinoma in situ (LCIS). Both forms are associated with an increased risk of developing invasive carcinoma. 25-30% of patients with DCIS and 20-30% of patient with LCIS develop invasive breast carcinomas within 15-20 years. (Haagensen *et al.*, 1978; Page *et al.*, 1982). However, in contrast, patients with DCIS will develop invasive carcinoma at the same site as the original DCIS (Page *et al.*, 1982) whereas only 20% of patients possessing LCIS develop invasive carcinomas at the same site. Indeed 50% of LCIS patients develop invasive carcinomas in a different breast than the original LCIS (Haagensen *et al.*, 1978). It is concluded from these observations that DCIS is a precursor to invasive breast carcinoma despite the absence of direct evidence. Lobular carcinoma is seen more as a marker for breast cancer than an intermediate stage.

1.1.3 Incidence and epidemiology of breast cancer.

Breast cancer is the most frequent malignancy diagnosed in women. It is thought that nearly one in twelve women in the western world will be affected by the disease at some point in their lifetime. An estimated 572,100 cases of breast cancer occurred world-wide in 1980 (Parkin 1988). This figure represented 18.4% of all new cancer cases diagnosed in women alone and 9% of all new cancer cases. Estimates of the global incidence of breast cancer by the end of the century have been as high as 1,000,000 cases per year (Millar and Bulbrook 1986). Breast cancer accounts for 15-20% of all cancer deaths and between 2 and 5% of all deaths in developed countries of the world (Adami *et al.*, 1990).

Although there is a relative lifetime risk of approximately 8% of developing breast cancer, not all women are equally at risk. Epidemiological studies have identified groups of women whose

risk of developing breast cancer is above that of the average (Boyle and Leake 1988; Kelsey and Berkowitz 1988; Willett 1989; Adami *et al.*, 1990). An increased risk has been shown to be associated with increasing age, early menarche, late menopause, high body mass index, high fat and \ or energy intake, radiation exposure, previous incidence of certain types of benign breast disease and the occurrence of the disease in one or more relatives. However, with the exclusion of age, family history and hormonal involvement, numerous studies have not been able to draw consistent conclusions about the effects of various risk factors examined (Boyle and Leake 1988).

Through epidemiological analysis of geographic differences in breast cancer incidence it has been proposed that as much as 80% of all breast cancer cases can be attributed to non-genetic environmental factors and so is therefore theoretically preventable (Doll and Peto 1981). However, despite extensive epidemiological study, it is thought that only 25% of breast cancer cases can be accounted for by currently identified risk factors (Seidman 1983). In addition such studies have failed to identify a risk factor which can offer the potential for preventative measures (Doll and Peto 1981).

1.1.4 Multistep tumorigenesis.

Cancer cells are defective in cellular growth control and differ from normal cells in that they possess the ability to divide continuously and invade surrounding tissue. They are sometimes able to move from their site of origin and establish secondary colonies in other organs of the body, a process known as metastasis. It is only within the past 20 years that the processes behind tumour generation have been elucidated.

The hypothesis of multistep tumorigenesis has now been almost universally accepted. Tumours are believed to be monoclonal in origin, ie they arise from a single cell. The transition of a normal cell to a malignant clone is thought to occur in a series of steps. Each step confers on the cell a growth advantage enabling the cell to outgrow neighbouring cells, a process known as clonal expansion (Nowell 1976). The resulting increase in cell number increases the chance of a further alteration occurring in one of the progeny cells. Any clone obtaining an alteration conferring a further growth advantage will again be able to outgrow the surrounding cells, thereby becoming the

predominant clone. Successive series of clonal expansion are thought to occur until a clone showing the malignant phenotype is produced. Further alterations are thought necessary to confer metastatic ability onto the tumour cells (Hart and Saini 1992).

Cancer is an age related disease and the majority of human cancers increase in direct proportion with age. This observation is in keeping with a model of multistep tumorigenesis and suggests the likelihood that a cell will contain all the changes necessary to confer a malignant phenotype increases with time. The number of events necessary for the generation of human malignancies has been estimated from the age incidence of common cancers. Analysis of the kinetics revealed a requirement for 5 or 6 independent rate limiting events to occur in the formation of a tumour (Armitage and Doll 1954) although only as few as two events may be necessary for certain childhood malignancies (Haber and Housman 1991).

Advances in molecular biology have led to the identification of some of the alterations occurring in tumorigenic cells. These include large and small alterations at the DNA level, increased and decreased expression of a wide variety of genes, altered methylation patterns of DNA, the loss, gain, rearrangement and translocation of chromosomes and alteration in ploidy. Recent research has identified genes playing a key role in the generation of a malignant phenotype which have been shown to be altered by the mechanisms listed above. These genes are normally involved in the control of cell growth and proliferation and, depending on their function, have been classed into two groups: the dominantly acting oncogenes and the recessive tumour suppressor genes.

1.2 Oncogenes.

1.2.1 Oncogenes and breast cancer.

Oncogenes were first discovered through work on cancer retroviruses capable of producing neoplastic growth in infected animals (Varmus 1984). Analysis of the viral DNA revealed the genetic elements responsible for viral oncogenicity to be altered copies of cellular genes involved in cell growth and proliferation. Further oncogenes have been identified through analysis of translocation breakpoints and cell transformation by tumour derived DNA (Varmus 1984; Bishop 1985). Currently over 40 oncogenes have been identified and characterised and are known to

function as cellular growth factors, growth factor receptors, signal transducers, protein kinases and transcription factors (Bishop 1985; Seemayer and Cavenee 1989). In human cancers proto-oncogenes are converted to dominantly acting oncogenes by point mutation, chromosomal translocation or amplification. Examples include point mutation at codons 12, 13 and 61 in the oncogene H-ras; over-expression of the oncogene myc by the promoters of the immunoglobin heavy chain gene via translocation; and amplification of the oncogene c-erbB2/neu/Her2.

Activated oncogenes are known to play a part in the genesis of most solid tumours although the specific oncogenes involved vary between malignancies. A number of oncogenes have been implicated in the development of breast cancer and those showing the highest frequency of alteration in breast tumours are neu, myc, int-2 and ras.

1.2.2 The neu oncogene.

The neu oncogene was initially identified from transfection studies using DNA from a chemically induced rat neuroglioblastoma (Schechler *et al.*, 1984). It was also cloned by virtue of its homology to the v-erbB oncogene in avian erthyroblastosis virus and named cerbB2 and HER2 (King *et al.*, 1985; Semba *et al.*, 1985). Neu belongs to a family of epithelial growth factor receptors along with the human epithelial growth factor receptor (EGFR) or cerbB1 and cerbB3 (or HER3). The neu gene has been mapped to 17q21 and codes for a protein of 185kD (p185 neu).

The transforming oncogene identified in the rat neuroglioblastoma possessed a point mutation resulting in dimerisation of the protein and an increased and constitutive activity of the protein's intracellular kinase domain (Weiner *et al.*, 1989). An investigation for similar transforming mutations identified no such alterations in 100 breast tumours (Lemoine *et al.*, 1990). However amplification of the neu gene has been detected in breast tumours and shows good correlation with over-expression of the normal p185neu protein (Lacroix *et al.*, 1989; Miller at al., 1991). Identification of over-expression without amplification in approximately 10% of breast carcinoma samples indicates that other mechanisms of transcriptional deregulation can occur (Slamon *et al.*, 1987). The neu gene has been the most intensely studied oncogene in breast cancer because of the reported potential of neu amplification as a marker for shorter relapse time and shorter overall

survival in breast cancer patients (Gullick 1990; Perren 1991; Borg 1992). In addition the presence of neu amplification in DCIS of certain histological subtypes and the maintenance of gene amplification throughout tumour development indicates that it may be one of the earliest genetic alterations to occur in breast cancer (Van de Vijers *et al.*, 1988). Estimates of the frequencies of amplification/over-expression have ranged from 9% (Barnes *et al.*, 1988) to 33% (Clark and McGuire 1991) but the majority of studies utilising a large number of samples show a frequency between 14 and 23% (reviewed by Clark and McGuire 1991; Perren 1991; Borg 1992).

1.2.3 The int-2 oncogene.

The int oncogenes were first described because of their activation by the MMTV (Mouse Mammary Tumour Virus) promoter sequences in the development of mammary tumours in mice. Although the MMTV does not contain a transduced oncogene it was shown to be inserted in the mouse genome at specific sites, the two most frequent sites of proviral insertion being adjacent to the int-1 and int-2 genes (Nusse 1988). The human homologue of int-2 is a member of the fibroblast growth factor family and maps to chromosome 11q13 (Dickson *et al.*, 1990). Estimates for the frequency of int-2 amplification in breast carcinoma range from 9% to 23% (Lidereau *et al.*, 1988; Varley *et al.*, 1988; Zhou *et al.*, 1988; Adnane *et al.*, 1989; Ali *et al.*, 1989a; Theillet *et al.*, 1989; Tsuda *et al.*, 1989; Fantl *et al.*, 1990; Meyers *et al.*, 1990; Theillet *et al.*, 1990; Borg *et al.*, 1991).

However in contrast to neu oncogene amplification there is poor correlation between amplification of int-2 and over-expression of the int-2 protein (Liscia *et al.*, 1989; Theillet *et al.*, 1989; Fantl *et al.*, 1990). Amplification of the 11q13 chromosome band is not int-2 specific and has been estimated to encompass between 2-3 Mb of DNA (Tanigami *et al.*, 1992). Amplification usually includes the proto-oncogene hst-1, another member of the fibroblast growth factor family (Adelaide *et al.*, 1988), and BCL-1, a gene associated with chromosomal breakpoints in B-cell lymphoma. However the identification of tumours possessing amplification of the BCL-1 gene without coamplification of the int-2 gene (Thelliet *et al.*, 1990), int-2 gene amplification without BCL-1 and hst-1 amplification (Ali *et al.*, 1989a) and int-2 amplification without hst-1 amplification (Tsuda *et al.*, 1989) question the involvement of these genes in the selective advantage of 11q13

amplification. These observations could be explained by the involvement of an as yet unknown gene present in the 11q13 amplified region (Szepetowski *et al.*, 1992).

1.2.4 The c-myc oncogene.

C-myc is a nuclear oncogene which codes for a transcription factor protein. The c-myc gene is the cellular homologue of v-myc, the transforming gene of the avian myelocytomatosis virus CM29, and maps to chromosome 8q24 (Watson *et al.*, 1983). C-myc shows sequence homology with two other known oncogenes, N-myc and L-myc, although only c-myc is thought to be implicated in breast cancer (Field and Spandidos 1990). Amplification of L-myc has only been detected in a single intraductal breast carcinoma (Varley *et al.*, 1987). Estimates for c-myc amplification in breast tumours range from 4% to 41% (Escot *et al.*, 1986; Varley *et al.*, 1987; Biunno *et al.*, 1988; Bonilla *et al.*, 1988; Guerin *et al.*, 1988; Garcia *et al.*, 1989; Seshadri *et al.*, 1989; Tavassoli *et al.*, 1989; Tsuda *et al.*, 1989; Borg *et al.*, 1992a; Pavelic *et al.*, 1992). These figures probably underestimate the involvement of c-myc in breast tumorigenesis since studies have detected increased c-myc expression in approximately 70% of breast tumour samples (Escot *et al.*, 1986; Whittaker *et al.*, 1986). In the study by Escot *et al.* (1986) only 6/14 tumours with elevated c-myc expression showed corresponding amplification of the c-myc gene indicating other mechanisms of transcriptional deregulation.

1.2.5 The ras oncogenes.

The ras proteins are a family of signal transducers involved in the transmission of growth promoting signals from the exterior to the interior of the cell. Oncogene members of the ras family implicated in breast carcinogenesis include Harvey ras (H-ras), Kirsten ras (K-ras) and N-ras which map to chromosomes 11p15, 12p12 and 1p13 respectively (Barbacid 1987). The importance of the ras proteins has been difficult to determine due to the variation between reports on their expression in breast carcinomas. While it is generally agreed that the activation of any of the ras genes by mutation (Spandidos *et al.*, 1987; Biunno *et al.*, 1988; Rochlitz *et al.*, 1989) or by amplification (Theillet *et al.*, 1986; Varley *et al.*, 1987) is rarely seen, estimates of over-expression encompass a

wide range of figures (reviewed by Field and Spandidos 1990). No over-expression of the Ki-ras, N-ras or H-ras oncogenes was detected in studies by Theillet *et al.* (1986) and Tanaha *et al.* (1986). In contrast, increased expression of H-ras was shown to occur in all breast tumours analysed by Agnantis *et al.* (1986a, 1986b).

1.3 Tumour suppressor genes.

1.3.1 Tumour suppressor genes.

Oncogenes have been shown to play an important part in the genesis of hemopoetic malignancies and sarcomas, frequently in the form of chromosome translocations (Sandberg 1991). Cancers originating from epithelial tissue, which account for 80% of all cancers including breast, generally show oncogene activation at a frequencies between 15-30% (Bishop 1985). It is believed that the tumour suppressor class of genes play a more important role in the development of these cancers.

Tumour suppressor genes are involved in the negative regulation of cell growth and proliferation. Unlike proto-oncogenes, which are converted to a dominantly acting form by mutation and over-expression, it is the inactivation of tumour suppressor genes which contributes to the tumorigenic process. Initial evidence for the presence of recessive genes involved in tumorigenesis came from somatic cell hybrid studies. The observation that the fusion of normal (N) and tumour (T) cells generally produced non-tumorigenic (NxT) hybrids was taken as evidence for a recessive component in the development of cancer (Stanbridge 1976). Propagation of these non-tumorigenic hybrid lines gave rise to occasional tumorigenic revertants. Analysis of the revertants revealed that they had lost chromosomes originally possessed by the non-tumorigenic parent hybrid and it was deduced that these lost chromosomes harboured genes responsible for the suppression of the malignant phenotype in the original tumour cell. By monitoring the loss of chromosomes from non-tumorigenic hybrids it was possible to identify specific chromosomes, the loss of which correlated with the switch to malignancy. These chromosomes were postulated to carry one or more tumour suppressor genes (Harris 1988). Further chromosomes showing a tumour suppressor effect were identified by the introduction of a single wild type chromosome into a tumour cell by microcell

fusion. The formation of a non-tumorigenic hybrid indicated the presence of a tumour suppressor gene on the donor chromosome. Human chromosomes potentially harbouring tumour suppressor genes identified in this way include 1, 3p, 6, 9 and 11 although to date no tumour suppressor gene has been isolated using these types of technique (Klein 1987; Sager 1989).

1.3.2 Retinoblastoma.

The paradigm for tumour suppressor genes is demonstrated by the ocular tumour retinoblastoma. Retinoblastoma is an uncommon childhood tumour which affects approximately 5 in 100,000 children. It occurs in both inherited and sporadic forms, 40% of cases are hereditary or define a new germline mutation, the remaining 60% are sporadic. The majority of sporadic cases are unilateral while a high percentage of familial or germline retinoblastoma cases are bilateral (Knudson 1971). In the familial form about 50% of the offspring develop a tumour indicating the transmission of an autosomal dominant gene. Analysis of the number of eye tumours occurring in the hereditary cases and comparison of age incidence graphs between bilateral (hereditary) and unilateral (mostly sporadic) cases suggested that the formation of the tumour was dependent on two events. From these observations Knudson postulated his 'two hit' hypothesis (Knudson 1971). It was proposed that in the familial form the first event was inherited, and so is present in all the cells of the carrier, and the second event occurred somatically in the target retinal cell. In the sporadic form both events were proposed to occur somatically. In a further modification of the two hit hypothesis the two events necessary for retinoblastoma formation involved the alteration of two alleles of a particular gene as opposed to two alterations involving two different genes (Knudson 1978).

In an attempt to locate the gene or genes, chromosome spreads of somatic cells from familial retinoblastoma patients were examined for any aberrations. In a small number of cases interstitial deletions of chromosome 13 were identified which always involved the band 13q14 (Knudson *et al.*, 1976, Yunis and Ramsay 1978). Linkage to a known marker mapping to chromosome 13, the esterase D gene, was found in retinoblastoma families (Sparkes *et al.*, 1980; Sparkes *et al.*, 1983). These observations strongly implicated 13q14 as a region harbouring a gene involved in retinoblastoma development through loss of at least one allele. The loss of a copy of

chromosome 13 and absence of esterase D activity levels in a single retinoblastoma tumour were used to infer loss of both copies of the putative retinoblastoma gene (Rb1) (Benedict *et al.*, 1983). This implied that the development of retinoblastoma was dependent on the inactivation of two alleles of the same gene and a variety of mechanisms were proposed in which recessive mutations could be unmasked (Cavenee *et al.*, 1983) (Figure 1.2).

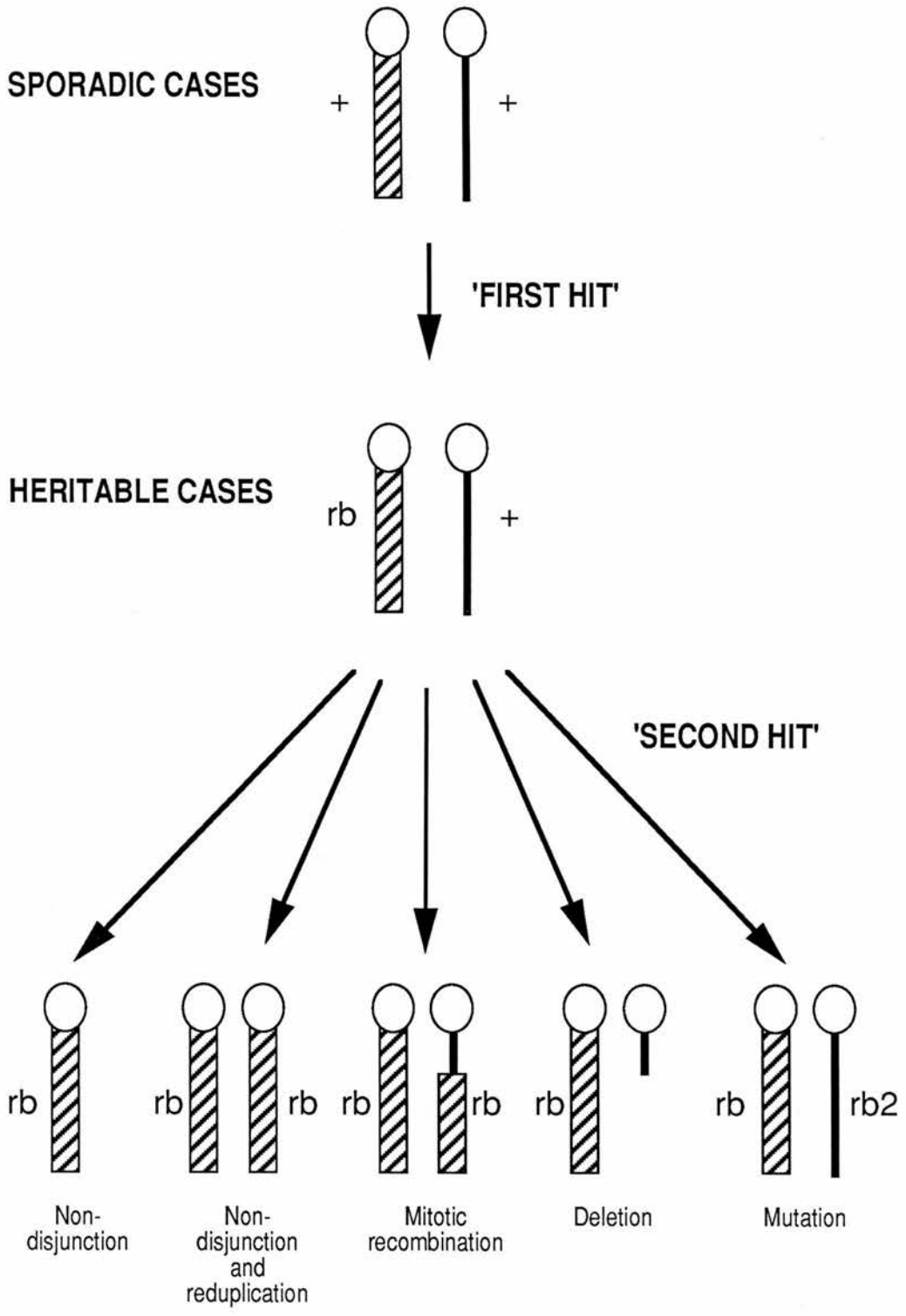
Although the familial retinoblastoma is seen to be transmitted as a mendelian dominant trait this definition is misleading, The retinoblastoma gene is recessive at a cellular level and the chance of inactivation of the second Rb1 allele in one of the 10^6 retinal cells in a familial patient is so high that retinoblastoma formation nearly always occurs. Therefore the trait appears to behave as a mendelian dominant. However it is only the susceptibility to retinoblastoma that is transmitted, an observation in keeping with the identification of occasional individuals with a constitutional mutant Rb1 allele who do not develop tumours (Gallie *et al.*, 1982).

The use of restriction length fragment polymorphisms (RFLPs) facilitated further investigation of the status of the Rb1 gene in retinoblastoma tumours (Cavenee *et al.*, 1983). RFLPs are a result of the variability naturally occurring in the human genome and are generated in two ways. An alteration in length of a particular DNA fragment after restriction digestion can occur either when a nucleotide base change creates or destroys a particular restriction enzyme site or when the DNA restriction fragment contains varying numbers of tandem repeat (VNTR) sequences (Nakamura *et al.*, 1987). RFLPs were utilised to identify sites on chromosome 13, and the two chromosome copies are distinguishable when they carry restriction fragments differing in length. Comparison of the chromosomal status between normal cells from retinoblastoma patients and the corresponding tumours revealed frequent reduction to homozygosity of a number of chromosome 13 markers in the tumours, indicating loss of genetic material including the putative Rb1 gene (Cavenee *et al.*, 1983).

1.3.3 The retinoblastoma gene.

Eventual cloning of the Rb1 gene was hampered because of the lack of a suitable positive selection technique. The search for new oncogenes has been facilitated by their dominant nature and

Figure 1.2



Potential mechanisms for the inactivation of both copies of the Rb1 gene in sporadic and hereditary retinoblastoma. The first hit (rb) is assumed to be a point mutation or small deletion and can be either somatically acquired or inherited.

for some (eg ras) cloning is relatively easy due to their transforming ability. Since the tumorigenic phenotype of tumour suppressor genes is only observed when both copies of the gene are inactivated the devising of a positive selection technique is difficult (Lee *et al.*, 1991). The introduction of an inactivated tumour suppressor gene into a normal or tumour cell would be expected to have no effect at all. The introduction of a wild type tumour suppressor gene into a tumour cell may lead to a loss of malignancy. However, in some cases the resulting reduction in growth would make cells harbouring the wild type gene difficult to isolate from a background of tumour cells (Eiden *et al.*, 1991).

Isolation of the Rb1 gene was achieved by undertaking a search of the 13q14 region by chromosome walking (Wicking and Williamson 1991). Beginning at the marker H38, a DNA fragment shown to be deleted in 3 out of 37 retinoblastomas, this technique was used to isolate adjacent DNA fragments in a bi-directional manner. The isolated fragments were tested for any cross-species homology, the presence of which would indicate a conserved sequence, in the hope of identifying an exon of a gene. A candidate sequence was identified which hybridised to an mRNA of 4.7kb in normal retinal cells and was absent or of an altered size in retinoblastoma cell lines (Friend *et al.*, 1986; Fung *et al.*, 1987; Lee *et al.*, 1987). The putative Rb1 gene was shown to be made up of 27 exons encompassing a region of over 200 kb and coded for a protein of 293 amino acids in length and 105kD in size (Bookstein *et al.*, 1988; Hong *et al.*, 1989). The sequence of the cDNA and protein have both been elucidated although no other sequence related to the RB1 gene has been found nor is there any other related protein of known function (Cowell 1991). The Rb1 protein is thought to regulate the activity of the transcription factors E2F and DRTF1, which in turn regulate cellular genes involved in growth and proliferation such as c-myc (Hamel *et al.*, 1992).

Analysis of the RB1 gene in retinoblastoma families and tumours indicated a wide variety of alterations at the DNA level, including large and small deletions (Fung *et al.*, 1987; Bookstein *et al.*, 1988; Canning and Dryja 1989; Hashimoto *et al.*, 1991) and missense and splicing point mutations (Dunn *et al.*, 1988; Horowitz *et al.*, 1989). Individuals carrying an inherited defect in the Rb1 gene have an increased risk of developing other malignancies, most notably osteosarcoma and soft tissue sarcomas (Draper *et al.*, 1986). Investigation of these tumours revealed frequent alteration

of the Rb1 gene (Friend *et al.*, 1987; Toguchida *et al.*, 1988; Reissman *et al.*, 1989; Stratton *et al.*, 1989; Wunder *et al.*, 1991). The retinoblastoma protein is expressed in a wide variety of normal tissues, indicating that it plays a role in cell growth regulation in many different cell types (Goddard *et al.*, 1988) and is found to be altered in tumours not normally associated with familial retinoblastoma susceptibility, eg tumours of the lung (Mori *et al.*, 1990; Murakami *et al.*, 1991a), prostate (Bookstein *et al.*, 1990a), oesophagus (Boynton *et al.*, 1991), liver (Murakami *et al.*, 1991b), central nervous system (Venter *et al.*, 1991), ovary (Li *et al.*, 1991), bladder (Horowitz *et al.*, 1989) and breast (Lee *et al.*, 1988, T'Ang *et al.*, 1988, Varley *et al.*, 1989, Borg *et al.*, 1992b).

Functional proof of a tumour suppressor gene is the suppression of the tumorigenic phenotype of malignant cells (Marshall 1991; Stanbridge 1992). Expression of the wild type Rb1 gene has been shown to reduce the growth rate and suppress the ability to form tumours of retinoblastoma and osteosarcoma cell lines (Huang *et al.*, 1988; Madreperla *et al.*, 1991). Tumorigenicity and/or growth were also reduced in bladder and prostrate carcinoma cell lines after transfection with the wild type Rb1 gene (Bookstein *et al.*, 1990b; Takahashi *et al.*, 1991; Goodrich *et al.*, 1992).

1.3.4 A model for tumour suppressor gene inactivation.

The identification of mutations in the germline of familial retinoblastoma patients (Yandell *et al.* 1989) and in sporadic retinoblastoma tumours (Canning and Dryja 1989) and the frequent loss of heterozygosity observed in sporadic retinoblastomas (Cavenee *et al.*, 1983; Dunn *et al.*, 1988; Zhu *et al.*, 1992) has contributed to the currently accepted model of tumour suppressor inactivation (Ponder 1988). In the model, the first allele is inactivated by a small alteration such as a point mutation or small deletion. The second copy of the gene is inactivated by an event effecting a much larger region of DNA such as chromosome loss or mitotic recombination. Evidence to support the sequence of events occurring in the progression of a tumour is limited as it requires the identification and isolation of sufficient tumour precursor cells for analysis.

The approach just described for tracking down the Rb1 gene has been used to isolate tumour suppressor genes involved in a variety of malignancies. The identification of regions potentially

harbouring tumour suppressor genes has relied on three avenues of investigation, cytogenetics, linkage analysis and loss of heterozygosity studies and will be discussed in the next section.

1.4 Isolation of tumour suppressor genes.

1.4.1 Cytogenetics.

The value of translocations in the identification of oncogenes involved in hemopoietic malignancies such as leukemias and lymphomas has been well documented (Sandberg 1991). Similarly the benefits of karyotyping normal cells from members of cancer families in order to locate tumour suppressor genes has been demonstrated. Identification of germline abnormalities by cytogenetic analyses has been instrumental in beginning the search for the WT1 gene (Francke *et al.*, 1979), the Rb1 gene (Knudson 1976; Yunis and Ramsay 1978) and the APC and MCC genes (Herrera *et al.*, 1986). Areas implicated by cytogenetics in sporadic solid tumours include chromosome 3p in small cell lung cancer, chromosome 9 in bladder carcinoma and chromosome 10 in prostate carcinoma (Sandberg *et al.*, 1988).

1.4.2 Linkage analysis.

Linkage studies rely on the fact that many of the common human cancers occur in a dominantly transmitted familial form (Knudson 1978). The inheritance of an inactivated copy of a tumour suppressor gene, as occurs in the inherited form of retinoblastoma, is thought to be the usual basis of familial cancer syndromes (Seemayer and Cavenee 1989). The aim behind linkage studies is to identify known markers so close to the disease locus that they co-segregate through generations with the susceptibility phenotype. Linkage studies are based on the LOD (log of the odds) score which is a measurement of the frequency at which the observed distribution of marker and disease susceptibility alleles would arise by chance (Emery 1976). A LOD score of over 3 is regarded as the standard for positive linkage, a LOD score of -2 or less is regarded as evidence against linkage at a stated recombination 'distance'. Information on the precise location of a putative tumour suppressor gene can be obtained by calculating the lod score at a number of sites on the same chromosome arm and by the analysis of any crossover events, which result in recombinant chromosomes and the non

co-segregation of the marker and the disease. Chromosomes implicated in familial cancers by linkage analysis include 3p in von-Hippel Lindau syndrome (Seizinger *et al.*, 1988), 5q in familial adenomatous polyposis (Bodmer *et al.*, 1987), 10 in multiple endocrine neoplasia type 2 (Simpson *et al.*, 1987), 11q in multiple endocrine neoplasia type 1 (Larsson *et al.*, 1988) and 17q in neurofibromatosis type 1 (Seizinger *et al.*, 1987).

1.4.3 Loss of heteozygosity studies.

Loss of heterozygosity (LOH) studies, comparing constitutional (usually blood) and tumour DNA from the same individual, are based on the assumption that the inactivation of tumour suppressor genes is often accompanied by a loss of genetic material. Extensive searches for LOH in a very wide variety of malignancies has been undertaken using probes detecting RFLPs in areas all over the genome (Ponder 1988; Sager 1989; Cavenee 1991). Areas which show a high frequency of LOH in a number of different tumour types include chromosomes 1, 3, 5, 11, 13, 17, 18 and 22. Following identification of a locus showing frequent loss, analysis of a number of nearby markers for LOH can be used to generate maps showing the extent of the region of loss for each tumour. By analysing the areas of loss occurring in a group of tumours a region showing loss that is common to each tumour, known as the shortest region of overlap (SRO), can be identified. In this way informative samples can lead to the narrowing down of the region potentially harbouring a tumour suppressor gene until the search for the coding sequence of the gene becomes feasible.

To date seven tumour suppressor genes have been characterised and six have been tracked down by using either linkage analysis (NF1 on chromosome 17q), loss of heterozygosity studies (WT1 on 11p, DCC on chromosome 18q) or both (MCC and APC on chromosome 5q and Rb1 on chromosome 13q).

1.5 The search for tumour suppressor genes involved in breast cancer.

1.5.1 Breast Cancer Cytogenetics.

Karyotypic analysis of normal cells from members of breast cancer families has revealed no chromosomal aberration associated with the transmisson of the disease. The gathering of cytogenetic

information on breast tumour samples has been hampered by practical and technical restraints common in many solid tumours. These include the low mitotic activity of tumour cells and their poor growth under *in vitro* culture conditions, low yields of tumour cells from fresh biopsy material, overgrowth of normal cells present in the tumour material and the fuzzy morphology of chromosomes seen in metaphase spreads (Sandberg 1988; Teyssier 1989).

Early work analysed clinical material such as pleural effusions, metastatic breast tumours or breast cancer cell lines which showed highly complex karyotypes featuring many chromosome alterations (Cruciger *et al.*, 1978; Teyssier 1989). It has been suggested that observations from pleural effusions and metastatic breast tumours are a consequence of sampling stages late on in the tumorigenic process and that secondary changes may well have occurred during the *in vitro* culturing of the cell lines (Trent *et al.*, 1985). Later studies, which relied on the analysis of cells taken either directly from the tumour or cultured through one or two passages, showed a reduction in the number and range of aberrations observed. However obtaining a sufficient quality and quantity of cells for analysis is a problem when working with direct preparations. Techniques in which samples are grown through a few passages on a plastic film may present a selective bias. The observation of predominantly diploid tumours when using this type of method (Zhang *et al.*, 1989; Geleick *et al.*, 1990; Wolman *et al.*, 1985) contrasts with cytometry and cytogenetic studies which indicate that the majority of breast tumours are aneuploid, or contain an aneuploid stemline as well as a diploid component (Devilee and Cornelisse 1990).

No cytogenetically identifiable alteration has been reported that is common to all breast tumours (Trent *et al.*, 1985; Mitchell and Santibanez-Koref 1990). Although comparison between studies is complicated by differences in technique and sample origin, many of the karyotype analyses identify specific chromosomes frequently involved in structural rearrangements. The majority of studies show that chromosome 1 is the most frequently involved in chromosomal aberrations including translocations, duplication, deletions and frequent iso-chromosome formation (Cruciger *et al.*, 1976; Brito-Babapulle and Atkin 1981; Rodgers *et al.*, 1984; Gerbault-Sereau *et al.*, 1987; Hill *et al.*, 1987; Dutrillaux *et al.*, 1990; Mitchell and Santibanez-Koref 1990; Ferti-Passantonopolou *et al.*, 1991). In a tumour sample described by Kovacs (1981) chromosome 1 was involved in 3 separate

marker chromosomes and 9 copies of the 1q arm were found per tumour cell. Of three aberrations found in a series of 40 diploid breast tumours and 5 breast metastases analysed by Zhang *et al.*, (1989) two were identified as translocations involving chromosome 1. In several studies specific bands or regions on chromosome 1 including 1q11-1q21 (Brito-Babapulle and Atkin 1981), 1q22-1q23 (Ferti-Passantonopoulou *et al.*, 1991) and 1qter-1q21 (Rodgers *et al.*, 1984, Hill *et al.*, 1987) have been found to be altered in a high proportion of samples. However cytogenetic analysis has not been able to define a region or band commonly involved in all these abnormalities.

A review of 113 breast tumour karyotypes by Mitchell and Santibanez-Koref (1990) calculated that of 304 chromosome bands in the genome 229 where involved in cytogenetic abnormalities. When the relative size of each band was taken into account only 5 bands, 1p11, 1p13, 1p22, 1q21 and 1q23 were shown to be involved at a frequency greater than that expected by chance. The lack of specificity in the chromosomal breakages and the involvement of chromosome 1 at a high rate in nearly all neoplasms investigated (Brito-Babapulle and Atkin 1981) may well reflect either chromosome instability or an effect, rather than a cause, of tumorigenesis. Other chromosomes implicated in a number of studies include 8 ,11, 13, 16 and 17 (Rodgers *et al.*, 1984; Ferti-Passantonopoulou *et al.*, 1987, 1991; Hill *et al.*, 1987; Dutrillaux *et al.*, 1990; Mitchell and Santibanez-Koref 1990). Interestingly these chromosomes are the location of known tumour suppressor genes and oncogenes. The oncogenes N-ras, ski, and ets map to 1p13, 1q23 and 11q22-23 respectively. The tumour suppressor genes Rb1 and p53 map to chromosomes 13q and 17p respectively. The loss of genetic material on chromosomes 8 and 16 may indicate as yet undiscovered tumour suppressor genes involved in human breast tumorigenesis (Rodgers *et al.*, 1984; Hill *et al.*, 1987; Dutrillaux *et al.*, 1990).

1.5.2 Linkage analysis in hereditary breast cancer.

It has been established from epidemiological studies that hereditary factors contribute to breast cancer development (Lynch *et al.*, 1984). Familial clustering of cancer is observed in 20% of breast cancer patients and segregation analysis indicates that a major susceptibility gene may account for this pattern in about 5-10% of breast cancer patients (Go *et al.*, 1983; Goldstein *et al.*,1987). A

single dominant autosomal gene with a penetrance of 57-92% best describes the observed patterns of these cases of inherited breast cancer (Williams and Anderson 1984; Bishop *et al.*, 1988; Newman *et al.*, 1988; Claus *et al.*, 1991). Hereditary breast cancer (HBC) is generally characterised as having an earlier onset than the sporadic form with an increased incidence of multiple primaries and bilateral involvement. HBC is heterogeneous and can occur as a site specific form, as well as in association with ovarian cancer; soft tissue sarcomas and leukaemia (SBLA or Li-Fraumeni syndrome); and gastrointestinal cancer and cutaneous manifestations (Lynch type II) (Lynch *et al.*, 1989).

Difficulties in the identification of families with HBC and the genetic heterogeneity of the condition have prevented linkage studies from contributing mapping information to the same extent that, for example, the study of the inherited colon cancer syndrome FAP has to the isolation of the APC gene on chromosome 5q (Groden *et al.*, 1991; Nishisho *et al.*, 1991). However, positive linkage scores have been reported for a number of sites including 1p22 (Hall *et al.*, 1989), 1p34-36 (Ferrell *et al.*, 1989), 6q24-27 (Zuppan *et al.*, 1991), 8q24-ter (King *et al.*, 1980; Ferrell *et al.*, 1989) and 16q (King *et al.*, 1980). Breast cancer susceptibility shows strong linkage to a site at 17q21 in both breast specific and breast-ovarian families (Hall *et al.*, 1990). Initially positive LOD scores were generated when considering early onset families only (Hall *et al.*, 1990). However reanalysis of the data indicates a potential role for the proposed 17q gene, named BRCA1, in late onset families (Margaritte *et al.*, 1992). Linkage to this site has been confirmed (Narod *et al.*, 1991; Porter *et al.*, 1993) and, using data from two studies, it has been possible to locate the BRCA1 gene to an interval of 4cM between the markers THRA1 and Mfd188 (Hall *et al.*, 1992; Bowcock *et al.*, 1993). Tumours from breast-ovarian patients inheriting the proposed mutant BRCA1 gene often show losses of the wild type chromosome 17q, suggesting that BRCA1 acts as a tumour suppressor (Smith *et al.*, 1992). An absence of linkage to the 17q21 locus, ascribed to disease heterogeneity, has been demonstrated in a number of cancer families (Sobol *et al.*, 1992; Haile *et al.*, 1993). Consequently, the BCRA1 has been estimated to be involved in almost all families with breast-ovarian cancer and half of those with site specific breast cancer (Easton *et al.*, 1993).

1.5.3 Loss of heterozygosity studies in breast cancer.

Areas of the genome shown to undergo LOH at greater than background levels have been used to indicate the location of tumour suppressor genes. Due to the unstable nature of chromosomes in cancer cells it is thought that all chromosome loci undergo LOH at a low background frequency. Those losses resulting in a growth advantage are selected for and will therefore be detected more frequently in LOH studies. From studies in colon cancer it has been suggested that a frequency of LOH above 25% should be taken as significant (Vogelstein *et al.*, 1989). Estimation of a background frequency has been attempted in breast cancer by determining the frequency of LOH in regions not thought to harbour tumour suppressor genes. A frequency of under 5% was found in two studies (Mackay *et al.*, 1988a; Chen *et al.*, 1989) suggesting that it is possible for a specific tumour suppressor gene to be inactivated in a subgroup of breast tumours at a frequency below 25%. Furthermore three studies investigating all chromosome arms for LOH in breast tumours detected a wide range of LOH frequencies occurring on various chromosome arms (Larsson *et al.*, 1990; Sato *et al.*, 1990; Devilee *et al.*, 1991a). It is therefore very difficult to determine an estimate for the frequency of background LOH and any figure would have to be an arbitrary one.

The frequency of LOH occurring at different regions of the genome in breast tumour samples has been determined by many independent groups (Ali *et al.*, 1987; Lundburg *et al.*, 1987; Mackay *et al.*, 1988a; Mackay *et al.*, 1988b; Ali *et al.*, 1989b; Chen *et al.*, 1989; Devilee *et al.*, 1989; Genuardi *et al.*, 1989; Borresen *et al.*, 1990; Coles *et al.*, 1990; Cropp *et al.*, 1990; Devilee *et al.*, 1990; Gendler *et al.*, 1990; Larsson *et al.*, 1990; Sato *et al.*, 1990; Thompson *et al.*, 1990; Chen *et al.*, 1991a; Davidoff *et al.*, 1991a; Devilee *et al.*, 1991a; Devilee *et al.*, 1991b; Devilee *et al.*, 1991c; Devilee *et al.*, 1991d; Leone *et al.*, 1991; Osborne *et al.*, 1991; Sato *et al.*, 1991a; Thorlacius *et al.*, 1991; Andersen *et al.*, 1992; Bieche *et al.*, 1992; Borg *et al.*, 1992b; Borg *et al.*, 1992c; Cheickhi *et al.*, 1992; Chen *et al.*, 1992; Futreal *et al.*, 1992; Hovig *et al.*, 1992; Kallioniemi *et al.*, 1992; Matsumura *et al.*, 1992; Sato *et al.*, 1992; Takita *et al.*, 1992; Thompson *et al.*, 1992; Cornelis *et al.*, 1993; Knyazev *et al.*, 1993). Data produced from these studies can be used in two ways. First, to determine the importance of a particular region of the genome in breast cancer development. Loci showing a high rate of loss are potentially more important than those showing a lower rate.

Areas of the genome showing LOH in breast tumours at a frequency of greater than 20% include 1p, 7q , 8p, 8q, 9q, 11q, 13q, 15q and 22q and those showing a frequency greater than 50% include 1q, 3p, 6q, 16q, 17p, 17q and 18q. Second, LOH data can be used in the isolation of putative tumour suppressor genes by the determination of a shortest region of overlap (SRO). Studies using more than one marker on a single chromosome arm can often define a SRO region from samples showing discordant LOH status between adjacent markers. Progress in this field has been achieved on several chromosome arms and will be outlined in the next section.

1.5.4 Chromosome 1.

Initial LOH findings on chromosome 1 indicated two regions of loss which could be narrowed down to 1p34 - 1pter (Genuardi *et al.*, 1989) and 1q23 - 1q32 (Chen *et al.*, 1989). Two further studies indicated that a region proximal to 1q24 - 31 (Devilee *et al.*, 1991b) and 1q42 - 43, possibly near to the polymorphic epithelial mucin (PEM) locus at 1q21 (Gendler *et al.*, 1990), may harbour a tumour suppressor gene. A SRO mapping between 1p33 - 35 and 1p36 has also been determined (Borg *et al.*, 1992c). The two initial investigations report that LOH occurs by means of a simple deletion in all cases examined (Chen *et al.*, 1989; Genuardi *et al.*, 1989) while a gain in allele intensity, suggesting an increase in chromosome copy number or sequence amplification, is reported at a low frequency by Gendler *et al.* (1990) and at a much higher rate by Devilee *et al.* (1991b), Borg *et al.* (1992c) and Cheickhi *et al.* (1992). These 'gain of allele' data may reflect cytogenetic observations of over-representation of chromosome 1 in many breast cancer samples (section 1.5.1). It has been suggested that this duplication of chromosomal material is due to the activation of one of several oncogenes located on chromosome 1 and that the complex rearrangements observed in breast tumours may arise through different selective pressures operating on different regions of the chromosome (Gendler *et al.*, 1990).

1.5.5 Chromosome 3p.

Analysis of LOH patterns on the short arm of chromosome 3 have determined two regions of loss. One SRO defines an area encompassing 3p21 to 3p24 - 25 (Ali *et al.*, 1989b) while another

more proximal SRO maps between 3p13 and 3p14.3 (Sato *et al.*, 1991a). Members of the c-erb growth receptor family and the protein tyrosine phosphatase gene, which map to chromosome 3p, have been suggested as possible candidate genes although their role in breast tumorigenesis has yet to be investigated (Ali *et al.*, 1989b; Croce 1991).

1.5.6 Chromosome 11p.

A single SRO was determined by Ali *et al.* (1987) which implicated the area between the β-globin gene at 11p15.5 and the parathyroid hormone (PTH) gene at 11p15.4. A further two studies were unable to confirm this result. LOH mapping revealed a number of tumour samples with complex patterns of LOH and a single SRO could not be identified (Mackay *et al.*, 1988b; Devilee *et al.*, 1989). However breast tumour samples showing LOH between 11p15.5 and 11pter and proximal to 11p15.5 have been identified suggesting two areas of chromosome 11p as potential tumour suppressor regions (Devilee *et al.*, 1989). Discordant LOH status observed between markers at 11p15.5-ter and 11p15.4-15.5 in 28 informative tumours suggested a tumour suppressor gene located in the peritelomeric region of chromosome 11p, distal to 11p15.4-15.5 (Takita *et al.*, 1992).

1.5.7 Chromosome 13q.

LOH was first identified on chromosome 13 in 4/10 ductal breast tumours by Lundberg *et al.* (1987). No breakpoints were identified although a further study by Devilee *et al.* (1989) revealed an SRO between the marker x13q12-13q14 and p68SR, a marker mapping to the internal part of the RB1 gene at 13q14, strongly suggesting a role for the Rb1 gene in breast cancer (Devilee *et al.*, 1989).

Rearranged Rb1 genes were found in 2/9 breast cancer cell lines accompanied by loss of protein expression (Lee *et al.*, 1988). In a similar study 4/16 breast cancer cell lines and 3/41 breast tumour samples were shown to have structurally altered Rb genes resulting in the absence or truncation of the RB1 mRNA transcript (T'Ang *et al.*, 1988). Of 15/77 breast tumour samples showing structural alterations to the RB1 gene 14 had a corresponding absence of the protein in a proportion of tumour cells (Varley *et al.*, 1989). However, in a recent study no correlation was found

between allele loss at the Rb1 locus and reduced Rb1 protein levels suggesting that the reduction of protein is brought about by mechanisms other than allele loss or that perhaps LOH on chromosome 13 contributes to the inactivation of another tumour suppressor gene adjacent to the Rb1 gene (Borg *et al.*, 1992b). No mutations have been detected in exon 21 of the Rb1 gene in 11 breast carcinomas, an exon previously shown to be mutated in prostate and bladder cancers (Hovig *et al.*, 1992).

1.5.8 Chromosome 16q.

LOH on chromosome arm 16q has been detected in 40-57% tumour samples (Larsson *et al.*, 1990; Sato *et al.*, 1990; Devilee *et al.*, 1991a). Detailed mapping of chromosomal deletions reveals a SRO between loci at 16q22.1 and 16q22 - 23 (Sato *et al.*, 1990).

1.5.9 Chromosome 17p.

Following the discovery of a high incidence of LOH in colon carcinomas a single SRO was determined between 17p12 and 17p13.3. Due to the tumour suppressing properties and chromosomal position of the p53 gene it was selected as a candidate gene and subsequently found to be mutated in colon tumours at a high frequency (section 1.6). Consequently many groups have analysed breast tumour samples for LOH at chromosome 17p loci (detailed in chapter 3) and p53 mutation (detailed in chapter 5).

1.5.10 Chromosome 17q.

A low frequency of LOH was detected on chromosome 17q in initial studies (Mackay *et al.*, 1988a; Devilee *et al.*, 1989; Sato *et al.*, 1990). The use of additional probes covering the 17q arm revealed LOH in 30-40% of tumour samples (Cropp *et al.*, 1990; Larsson *et al.*, 1990; Thorlacius *et al.*, 1991; Sato *et al.*, 1991a; Devilee *et al.*, 1991a; Andersen *et al.*, 1992; Futreal *et al.*, 1992; Knyazev *et al.*, 1993). Information on the potential location of the putative tumour suppressor gene was determined by several groups (Cropp *et al.*, 1990; Sato *et al.*, 1991a; Andersen *et al.*, 1992; Futreal *et al.*, 1992). By combining these maps the SRO has been narrowed down to the

chromosome band 17q21, the site of the BRCA1 gene. Using this localisation information an anti-proliferative gene called prohibitin, which shows homology to the tumour suppressor gene NF1 and maps to 17q21, was analysed in breast tumour samples. In 23 tumours analysed 4 tumours possessed an altered prohibitin gene with a corresponding LOH on chromosome 17q (Sato *et al.*, 1992). Unfortunately, these findings have not been corroborated elsewhere (Skolnick *et al.*, unpublished data). Recent data generated from 12 loci on chromosome 17q revealed complex patterns of LOH, suggesting the presence of at least two putative tumour suppressor genes on chromosome 17q (Cornelis *et al.*, 1993).

1.5.11 Chromosome 18q.

The loss of chromosomal material on chromosome 18q in breast cancer is of special interest as a known tumour suppressor gene, DCC, is known to map to 18q21 (Fearon *et al.*, 1990). In a study by Cropp *et al.* (1990) a high frequency of LOH was detected on chromosome 18q with an SRO defining an area between 18q21.3 and 18q33, implicating DCC in breast cancer. However a probe mapping to the DCC locus detected LOH in only 1 of 40 (2.5%) tumour samples in a study by Sato *et al.* (1991a) and no common region of loss was found by Devilee *et al.* (1991c). Further investigation of the expression and gene alteration of the DCC gene in breast tumours needs to be carried out before the DCC gene can be excluded from a role in breast cancer development.

1.5.12 Specificity of LOH to breast cancer.

Although loci on chromosomes 1p, 1q, 3p, 11p, 13q, 17p, 17q, and 18q have been shown to undergo LOH in breast tumours in several studies, none of these areas appear to be breast cancer specific. For example loci at 3p frequently undergo LOH in lung cancer and linkage to this area has been shown for von-Hippel Lindau syndrome, which features a familial form of adrenal cancer. This supports the observation that any one tumour suppressor gene may be involved with more than one type of malignancy and that a breast tumour specific tumour suppressor gene may not exist. This is advantageous since mapping information from several malignancies could potentially be used in the isolation of tumour suppressor genes involved in breast tumorigenesis.

1.6 The p53 gene

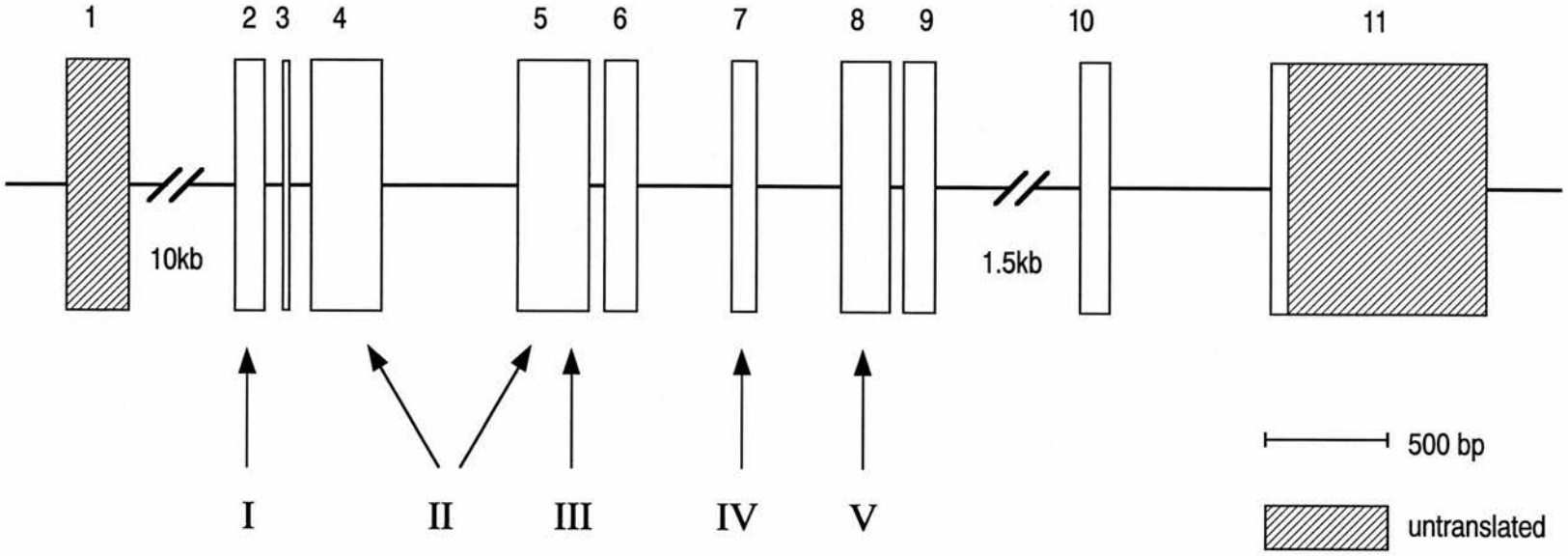
1.6.1 Isolation and characterisation of the p53 gene.

p53 was first identified as a cellular protein able to form stable complexes with the viral SV40 T antigen in SV40 transformed cells (Lane and Crawford 1979). Antibodies directed against the p53 protein were frequently detected in animals with SV40 induced tumours and in those immunised with chemically transformed MethA cells (De Leo *et al.*, 1979; Linzer and Levine 1979). The p53 protein was classified as a tumour antigen following the discovery of sera containing anti-p53 antibodies in 10% of humans with cancer (Crawford *et al.*, 1982; Caron de Fromental *et al.*, 1987). The first cDNA clone for the murine p53 gene was isolated by an RNA enrichment technique (Chumakov *et al.*, 1983) and subsequent clones were isolated and sequenced from a variety of tissue types. The human p53 gene was cloned through homology with the mouse gene (Matlashewski *et al.*, 1984; Harlow *et al.*,1985).

The p53 gene has been intensely studied in the mouse and human where it maps to chromosome 11 (Czonek *et al.*, 1984; Rotter *et al.*, 1984) and chromosome 17p13.1 respectively (Benchimol *et al.*, 1985; Isobe *et al.*, 1986; McBride *et al.*, 1986). The human p53 gene is comprised of 11 exons and 10 introns spanning approximately 20kb of DNA and codes for a 2.5kb mRNA transcription product which is translated into a protein of 393 amino acids (Harlow *et al.*, 1985; Lamb and Crawford 1986) (see figure 1.3).

Little is known about the promoter/enhancer region of the p53 gene. Two transcription initiation sites have been mapped to the 5' end of the p53 gene. The first, p53p1, maps immediately upstream of the first exon, and the second, p53p2, is located approximately 1kb downstream from exon 1 (Reisman *et al.*, 1988). p53p2 has been shown to promote transcription 20 times more efficiently than p53p1 and transcripts from both sites have been identified *in vivo* (Reisman *et al.*, 1989). The p53p1 regulatory sequences have been shown to be highly conserved between human and mouse but are unusual as they do not contain any of the consensus sequences e.g. TATA, CAAT found in most eukaryotic regulatory elements (Bienz-Tadmor *et al.*, 1985; Lamb and Crawford, 1986).

Figure 1.3



Genomic organisation of the human p53 gene.
Exons 1 - 11 are shown as boxes, introns as single lines. The positions of the five highly conserved domains are indicated by the markers I - V.

1.6.2 Evolutionary conservation of the p53 gene.

The p53 gene has been identified only in vertebrate species despite many efforts to detect an invertebrate copy of the gene (Soussi *et al.*, 1990; Nigro *et al.*, 1992). Initial comparison of the murine with human and Xenopus p53 protein sequences revealed an 81% and 57% homology respectively (Soussi *et al.*, 1987). The conservation of sequence is not spread evenly throughout the molecule (Soussi *et al.*, 1990) and five areas of high homology, ranging from 91-100%, have been identified. These five domains, I, II, III, IV and V are positioned between codons 13-19, 117-142, 170-181, 234-258 and 270-286 respectively (Soussi *et al.*, 1990). The conserved domains are thought to play a central role in the function of the protein since the sequences flanking these domains show much greater divergence, especially in the N-terminal region (Soussi *et al.*, 1990). Additional p53 sequences from rat (Soussi *et al.*, 1988a), chicken (Soussi *et al.*, 1988b), monkey (Rigaudy and Eckhardt 1989) and trout (Caron de Fromentel *et al.*, 1992) have been determined, all of which show a high degree of homology in domains I to V (Soussi *et al.*, 1990).

All the p53 proteins have a similar predicted structure consisting of three hypothetical regions differing in charge, hydrophobicity and structure (Pennica *et al.*, 1984; Levine and Momand 1990). The N-terminal region of the human p53 protein (residues 1-80) has a high proline content, contains a high number of acidic residues and very few basic residues. Structurally it is composed of a simple α-helix and carries an overall negative charge. The central region (residues 80-150) are arranged in a β-sheet formation containing few charged residues and three highly hydrophobic domains which correspond to the conserved domains II, III and IV. The carboxyl end of the protein (residues 319-393) is a negatively charged, very hydrophilic α-helix. A simple structure has been predicted for the p53 protein consisting of the central region forming a hydrophobic core with the amino and carboxyl terminal regions at the protein surface.

1.6.3 Expression of the p53 gene.

mRNA studies have shown that the p53 gene is expressed at low levels in most tissues though increased levels of expression have been detected in the spleen, the thymus and during mouse foetal development (Jenkins *et al.*, 1984; Rogel *et al.*, 1985). Proliferating cells show significantly

higher levels of p53 mRNA compared with resting G0 cells (Reich and Levine 1984) and high levels of p53 protein have been found in many transformed cell lines and tumour tissues. Since the p53 mRNA levels found in transformed cell lines and tissues are often comparable with normal tissue or non-transformed cell counterparts (Matlashewski *et al.*, 1984) it is thought that the regulation of p53 occurs in most cases at a post-translational rather than a transcriptional level (reviewed by Levine *et al.*, 1989). The regulation of cellular levels of the p53 protein is thought to be achieved by protein degradation, possibly by an ATP dependent proteolytic pathway (Gronostajski *et al.*, 1984). p53 protein found in untransformed cells has a short half life ranging from 6-30 minutes (Jenkins and Sturzbecher 1988). In contrast, transformed cells frequently contain a more stable p53 protein with an increased half life of up to forty hours resulting in a 100-1000 fold increase in the cellular level of the protein (Levine *et al.*, 1988).

1.6.4 Biological properties of the p53 gene.

Genomic and cDNA clones from various sources were used to demonstrate that the p53 gene could immortalise rat chrondrocytes (Eliyahu *et al.*, 1984) and, in co-operation with the ras oncogene, could elicit full cell transformation in rat embryo fibroblasts (Jenkins, 1984; Parada *et al.*, 1984). Due to the positive transforming ability and nuclear localisation of the protein (Dippold *et al.*, 1981; Rotter *et al.*, 1983) the p53 gene was classified as a nuclear oncogene and grouped with the myc, myb, fos and E1a genes. However, work on Friend erythroleukemia virus infected mice demonstrated that malignant tumours frequently contained structurally rearranged p53 genes resulting in the loss of p53 expression altogether (Mowat *et al.*, 1985). Similarly two tumorigenic cell lines were shown to express no p53 protein due to the inactivation of the p53 alleles by deletion and viral insertion (Wolf *et al.*, 1984; Wolf and Rotter 1985). Both of these observations are more in keeping with the role of the p53 gene as a tumour suppressor rather than an oncogene.

Further work demonstrated that the p53 gene sequences used in the transformation experiments, many of which had been isolated from transformed cell lines, were mutant versions of the wild type gene (Finlay *et al.*, 1988; Levine *et al.*, 1989). When these experiments were repeated using the wild type p53 gene no transforming potential was observed, suggesting that the activation

of the p53 gene occurred by mutation and not over-expression (Finlay *et al.*,1988; Hinds *et al.*, 1989; Mercer *et al.*, 1990). However the wild type p53 gene was found to suppress the transformation of rat embryo fibroblasts by mutant p53, myc and the viral oncogene E1a (Finlay *et al.*, 1989). Transformed foci were produced in low numbers following transformation with the wild type p53 gene. However, these cells expressed either no p53 protein or an altered form of the protein, indicating that oncogene mediated cell transformation was incompatible with wild type p53 expression (Finlay *et al.*, 1989).

1.6.5 Alteration of the p53 gene in human malignancies.

Following the newly discovered tumour suppressor properties of the p53 gene and the high frequency of LOH observed on chromosome 17p in colon cancer (Vogelstein *et al.*, 1988) the p53 gene was selected as a candidate tumour suppressor gene involved in colon tumorigenesis. Investigation of the locus revealed mutation of the p53 gene, and accompanying loss of the remaining allele, in a high proportion of colon carcinomas, a pattern in keeping with the inactivation of a tumour suppressor gene (Baker *et al.*, 1989; Baker *et al.*, 1990a). A similar pattern of mutation and accompanying LOH was determined following studies of the p53 gene in a wide variety of tumour types (Nigro *et al.*, 1989). Oncogene activation often involves a specific mutational change and the diversity of alterations of the p53 gene detected in sporadic tumours, including missense and nonsense mutations, deletions and insertions at many different sites in the gene provided indirect evidence that the p53 gene was undergoing a loss of function rather than a gain. In response to these findings numerous studies demonstrated a reduction in growth rate and/or tumorigenicity following the introduction of the wild type p53 gene into a variety of human cancer cell lines. No reduction was observed in parallel experiments using mutant forms of the p53 gene (Baker *et al.*, 1990b; Mercer *et al.*, 1990; Casey *et al.*, 1991; Chen *et al.*, 1990a; Chen *et al.*, 1991b; Shaulsky *et al.*, 1991; Cheng *et al.*, 1992; Takahashi *et al.*, 1992).

1.6.6 Properties of mutant p53 alleles.

Many lines of evidence suggested that the wild type p53 gene functions as a tumour suppressor. However, if p53 mutants were simply inactivated versions of the wild type gene they would not be expected to be capable of transforming of rat embyro fibroblasts. Mutant p53 genes would either have to behave as dominant oncogenes or in some way be able to inactivate the suppressive influence of the endogenous wild type protein. It has been proposed that mutant p53 can act in a dominant negative manner, whereby the mutant protein inactivates the wild type protein by binding to form heterologous oligomers (Herskowitz 1987). p53 forms tetrameric complexes in solution (Stenger *et al.*, 1992) and heterologous oligomers have been observed between mutant and wild type p53 proteins (Milner and Medcalf 1991). Experiments cotranslating both wild type and mutant p53 genes have shown that certain mutant p53 proteins are able to bind the wild type protein and drive it into a structural conformation usually associated with mutant proteins (Milner and Metcalf 1991). Although this mechanism can explain the dominant effect of p53 mutants, the dominant nature of some p53 alleles cannot be accounted for by the inactivation of the wild type protein. The most compelling example of this is the enhanced tumorigenicity of the cell line L12 following the introduction of a mutant p53 gene (Wolf *et al.*, 1984). Since L12 contains no endogenous p53 this effect cannot be explained as a consequence of the inactivation of wild type protein and suggests that the mutant p53 protein has acquired a true 'gain of function'.

The analysis of the p53 gene has lead to the realisation that the terms oncogene and tumour suppressor gene are not mutually exclusive and both properties can be attributed to the same gene. The wild type p53 gene can be termed as a tumour suppressor but classification of the various p53 mutants appears to be dependent on the specific sequence alteration (Michalovitz *et al.*, 1991). Mutants can theoretically be split into three groups: null mutants, which simply represent an inactivated form of the protein; the negative dominant mutants, which have the ability to interfere with the function of wild type p53 protein and would only be classed as oncogenic in cells that co-expressed the wild type p53 protein; and positive dominant mutants, which represent a true gain of function, and the oncogenic nature of which is independent of the presence of wild type protein.

Variation has become apparent following the isolation and analysis of more mutant p53

genes. Individual p53 proteins have been shown to differ in several biological properties including heat shock protein 70 (hsp70) binding, transforming efficiency, immunogenicity and half life (Halevy *et al.*, 1990; Hinds *et al.*, 1990; Bodner *et al.*, 1992; Davidoff *et al.*, 1992a; Winter *et al.*, 1992). For example, while colon carcinomas show frequent mutation at codons 175 and 273, the 175 His and 273 His p53 proteins have been shown to differ in transforming ability, protein conformation and half life (Levine *et al.*, 1991).

Alternatively, it has been proposed that the transformation properties of mutant p53 genes could be due to the high copy number of the introduced p53 alleles (Wang *et al.*, 1993). Three studies using retro-virus mediated gene transfer have shown that the introduction of a single wild type p53 gene is sufficient to reduce the tumorigenicity of breast, rhabdomyosarcoma and osteosarcoma cell lines (Chen *et al.*, 1990a; Chen *et al.*, 1991b; Wang *et al.*, 1993). The suppression of the malignant phenotype by the exogenous wild type p53 protein was shown to occur despite the 10 fold higher quantity of endogenous mutant p53 protein present in the cells suggesting that the maintenance of a malignant phenotype is dependent of the removal of the p53 tumour suppressor function in these cells.

1.6.7 Function of p53

p53 expression in normal cells appears to be cell cycle related and early evidence suggested that the p53 protein acts at the G0/G1 phase of the cell cycle. An increase in the level of p53 mRNA is observed in non-dividing mouse T-lymphocytes following mitogen treatment (Milner and Milner 1981). Mouse 3T3 fibroblasts arrested in G0 show a dramatic increase in p53 mRNA and protein during late G1 following serum stimulation (Reich and Levine 1984). Micro-injection of anti-p53 antibodies into serum starved G0 cells leads to the failure of the cells to enter G1 (Mercer *et al.*, 1982). Cells expressing anti-sense p53 RNA are compromised for growth (Shobat *et al.*, 1987) and over-expression of the wild type protein blocks cell division in the G1 phase of the cell cycle (Michalovitz *et al.*, 1990). These observations suggest that the p53 protein plays a critical role in the passage through G1 and therefore would be expected to be essential for cell viability. However,

mice deleted for both copies of the p53 gene are developmentally normal, although they develop a variety of tumours before they reach 6 months (Donehower *et al.*, 1992).

Sequence motif analysis has failed to elucidate a possible function of the p53 protein (Soussi *et al.*, 1990). At present there are two hypotheses to explain the function of the p53 protein. The first suggests that the protein is a transcription factor which regulates the expression of genes involved in growth. p53 appears to bind to a specific sequence (Kern *et al.*, 1991). A consensus p53 binding sequence consisting of two copies of a 10 base pair motif separated by 0-13 base pairs has been determined (El-Deiry *et al.*, 1992). Wild type p53 has been shown to increase the expression of a reporter gene containing 3.3 kb of the upstream control sequence of the muscle-specific creatine kinase gene (MCK), which is known to contain the proposed p53 specific binding sequence (Wientraub *et al.*, 1991, Levine 1992). In addition wild type p53 is known to inhibit the expression from the interleukin 6, β-actin, fos, major histocompatibility complex (MHC) class I (Santhanam *et al.*, 1991) and multidrug resistance (MDR1) promoters (Chin *et al.*, 1992). In both of these studies p53 mutants showed a loss or reduction in their ability to repress transcription. The highly acidic n-terminal region of the protein has been shown to activate transcription when coupled to the GAL4 DNA binding domain, a function lost in p53 mutant proteins (O'Rourke *et al.*, 1990; Fields and Jang 1990; Raycroft *et al.*, 1990; Unger *et al.*, 1992). Similarly, wild type p53 protein, but not tumour derived mutants, has been shown to transactivate genes adjacent to a p53 DNA binding site (Kern *et al.*, 1992). Furthermore, deletion of the c-terminal region of the protein abolishes the ability of the protein to bind genomic DNA (Frood *et al.*, 1992) suggesting that the DNA binding site is located in this region.

The second hypothesis suggests that the p53 protein is a regulator of replication or is a part of the replication initiation complex. p53 is known to bind to SV40 T antigen, which is required in the initiation of viral replication. Since viral genes often encode proteins that have a cellular homologue, p53 has been proposed to regulate the cellular SV40 T homologue, a protein presumably involved in DNA replication. In support of this hypothesis the p53 protein has been localised at sites of viral replication in herpes infected cells (Wilcock and Lane 1991) and the wild type p53 protein has been shown to bind to DNA sequences adjacent to the replication origin of the SV40 virus, an

ability that has been lost in mutant forms of the p53 protein (Bargonetti *et al.*, 1991). Although these two hypothesis propose two different functions for p53 the fact that some origins of replication are activated by the act of transcription near or at the origin may indicate that p53 has a dual function within the cell (Levine 1992).

Whatever the function of the p53 protein the effects of the inactivation or activation of the protein would seem to be far reaching. Mutant forms of the protein have been shown to increase metastatic potential (Pohl *et al.*, 1988), increase tumorigenicity (Wolf *et al.*,1984) and confer growth factor independence (Gai *et al.*, 1988) in transformation experiments. In addition, wild type p53 has been postulated to involved in the control of apoptosis (Yonish-Rouach *et al.*, 1991) and as a monitor of DNA damage (Lane 1992).

1.6.8 Heritable p53 mutations.

Hereditary breast cancer is estimated to account for 10% of breast cancer cases and can be associated with a number of other tumour types (section 1.5.2). Li-Fraumeni syndrome (LFS) is a rare familial cancer syndrome which features a spectrum of malignancies including breast, brain and adrenocortical carcinomas, osteosarcomas, soft tissue sarcomas and leukaemia (Li *et al.*, 1988). Breast cancer is the most frequent malignancy in the syndrome although the number of breast tumour patients with LFS is estimated to be less than 0.001% (Vogelstein 1990). LFS family members present with tumours at an early age, fifty percent will have cancer by the age of 30 and greater than 90% will develop cancer by the age of 70 (Malkin *et al.*, 1990). The syndrome best fits an inheritance model of a single dominant autosomal gene and the location of the gene responsible was hampered by the rarity of the syndrome, the high mortality of affected LFS individuals and the inability to distinguish between the hereditary tumours and those sporadic tumours arising in unaffected relatives. Unlike familial retinoblastoma and adenosis polyposis coli (APC) linkage studies and karyotypic analysis were unable to determine a possible location for the gene. Since tumour suppressor genes are closely associated with familial cancer syndromes and the p53 gene was known to be mutated in sporadic tumours of the types occurring in the Li-Fraumeni syndrome, it was selected as a candidate gene. Consequently p53 germline mutations were found in Li-Fraumeni

kindreds in a number of studies (Malkin *et al.*, 1990; Srivastava *et al.*, 1990; Santibanez-Koref *et al.*, 1991; Toguchida *et al.*, 1992; Felix *et al.*, 1992a). All LFS family members analysed are heterozygous for the p53 mutations and, in keeping with the tumour suppressor hypothesis, a number of studies report the loss of the wild type p53 allele in a number of tumours (Malkin *et al.*, 1990; Borresen *et al.*, 1992; Srivastava *et al.*, 1992; Sameshima *et al.*, 1992a).

1.7 Aims of the project.

Genetic alterations detected in breast tumours include the over-expression and amplification of oncogenes such as neu, int-2, c-myc and ras and the loss of genetic material from specific chromosomal loci, indicative of the inactivation of tumour suppressor genes. This project aims to examine the loss of genetic material occurring at sites on chromosome 17p and is a continuation of the work of Mackay *et al.* (1988a), who detected a high frequency of LOH at a locus at 17p13.3 in a series of paired breast tumour/control DNA samples. LOH at other loci on chromosome 17 was investigated in breast tumour samples in the hope of defining a region containing a putative tumour suppressor gene and also to determine a role for the p53 gene, which maps to 17p13.1, in breast cancer development.

Chapter 2
Materials and methods

2.1 Clinical materials.

All tumour, blood and lymphoblastoid cell line DNAs used in the course of this project were obtained from a bank of matched control/tumour DNA samples established by Dr James Mackay. All patients had presented with palpable lumps and underwent either Patey mastectomy, wide local excision or wedge biopsy at either the Breast Unit Longmore Hospital, the Western General Hospital or Royal Infirmary of Edinburgh.

20ml of venous blood was collected from these patients and transferred to a sterile heparinised universal. Tumour samples were immediately frozen on dry ice after excision and stored at -70°C. DNA was extracted from tumour and blood samples as described in section 2.2. Lymphoblastoid cell lines were established from 10ml of blood by Dr C. M. Steel (Mackay 1989).

The diagnosis of breast cancer was confirmed and the greatest diameter of the excised tumour was accurately measured at the University of Edinburgh Department of Pathology. The tumours were classified into histological types (Page and Anderson 1988) and examined for in situ carcinoma. Lymph nodes were examined histologically for metastases.

One portion of the tumour was placed on ice and immediately assayed for oestrogen receptor protein concentration by Dr R. A. Hawkins (Lister Laboratories of the University Department of Clinical Surgery) by an adsorption assay using Dextran coated charcoal (Hawkins *et al.*, 1981). The oestrogen receptor protein concentration of tumour material from patients who had received the anti-oestrogen drug Tamoxifen was also examined by enzyme immunoassay (Leclerq 1984) using the ER-EIA Kit (Abbott Laboratories).

Information on patient age, tumour size, histological typing and lymph node involvement was obtained from the Edinburgh Department of Pathology. Information on menopausal status and oestrogen receptor protein levels was obtained from the Lister Laboratories. Information on family history was obtained from the Breast Cancer Unit at the Western General Hospital (Table 1).

For control DNA, placental material was collected from the Obstetrics and Gynaecology Department of the Western General Hospital and stored at -70°C until DNA extraction.

Table 2.1 Clinical and pathological data on patients and breast tumour samples used in this study.

Tumour Number	Size (cm)	Histological Tumour Type	Nodal Status	Patient Age	M/P Status	Family History	ER level (pg)
1	-	-	-	70	-	No	724
3	-	-	0/?	52	-	No	0
4	-	-	-	79	Post	No	220
6	3.2	Ductal/NST	10/23	62	-	No	789
7	2.8	Ductal/NST	0/7	34	Pre	No	2
8	3.2	Ductal/NST	1/6	65	Post	No	122
9	9.0	Ductal/NST	4/5	73	Post	No	-
10	2.8	Ductal/NST	0/10	50	-	No	21
11	2.0	Ductal/NST (M)	10/17	50	-	No	3
12	3.4	Ductal/NST (M)	10/17	50	-	No	4
13	3.8	Ductal/NST	0/9	59	Post	No	15
14	1.4	Ductal/NST (M)	1/17	44	Peri	Yes	51
15	3.0	Ductal/NST (M)	1/17	44	Peri	Yes	39
16	2.0 (B)	Ductal/NST	-	60	-	Yes	80
18	2.8	Ductal/NST (M)	3/10	79	Post	No	0
19	1.2	Carcinoid (M)	3/10	79	Post	No	-
20	1.0	Ductal/NST	0/3	72	Post	No	-
21	3.5	Ductal/NST	7/10	66	-	No	0
22	1.3	-	3/4	83	Post	No	0
24	1.9	Ductal/NST	0/15	59	Post	Yes	-
25	5.5	Lobular variant	5/10	70	Post	No	0
26	3.0	Ductal/NST	0/21	75	-	No	54
27	2.5 (B)	Ductal/NST	-	57	Post	No	364
28	-	-	-	-	-	No	-
29	1.8 (B)	Ductal/NST	2/4	34	Pre	No	36
30	1.0 (B)	Ductal/NST	-	55	Post	Yes	46
32	3.9 (B)	Ductal/NST	0/5	47	Post	No	1
33	2.0	Ductal/NST	10/17	50	-	No	71
34	2.2 (B)	Ductal/NST	0/4	66	Post	No	8
35	1.2 (B)	Tubular	0/3	66	Post	No	275
37	4.0	Squamous	-	66	Post	No	4
38	1.5	Ductal/NST	4/4	60	Post	No	236
39	2.5	Ductal/NST	2/3	46	Peri	No	31
40	7.5	Ductal/NST	4/7	49	-	No	1
41	1.5	Ductal/NST	0/3	52	Post	Yes	-
42	1.5	Ductal/NST	1/4	47	Peri	No	82
45	3.8	Ductal/NST	0/2	51	Pre	No	0
46	2.5	Tubular	2/5	59	Post	No	119
47	1.5 (B)	Ductal/NST	-	68	Post	No	61
48	2.6	Ductal/NST	0/12	62	Post	Yes	489
49	1.6	Mucoid	3/3	39	Pre	No	23
50	-	Ductal/NST	0/3	37	-	Yes	3
53	2.2	Ductal/NST	5/9	54	Post	No	150
54	2.8	Ductal/NST	-	55	Post	No	186
55	3.3	Ductal/NST	0/14	67	Post	No	0
56	2.0	Ductal/NST	0/1	75	Post	No	204
57	1.9	Ductal/NST	4/4	66	Post	No	79
58	1.0 (B)	Ductal/NST	1/6	57	-	No	-

Table 2.1 (continued)

Tumour Number	Size (cm)	Histological Tumour Type	Nodal Status	Patient Age	M/P Status	Family History	ER level (pg)
59	2.7 (B)	Ductal/NST	1/5	61	Post	No	536
60	2.2 (B)	Ductal/NST	-	59	Post	No	392
61	2.6 (B)	Ductal/NST	-	62	Post	No	225
62	- (B)	Ductal/NST	-	54	Post	No	371
63	3.8	Ductal/NST	0/3	33	Pre	No	44
64	1.7	Ductal/NST	1/3	50	Peri	No	1
65	2.0	Scirrhous	3/3	45	Post	No	76
67	2.2	Ductal/NST	0/4	50	-	No	92
68	1.5	Ductal/NST (M)	0/8	52	-	No	212
69	0.8	Ductal/NST (M)	0/8	52	-	No	199
70	1.2	Ductal/NST	0/2	50	Post	No	-
71	1.3 (B)	Ductal/NST	1/1	47	Post	No	436
72	1.7 (B)	Ductal/NST	0/3	56	Post	No	658
74	- (B)	-	-	62	Post	No	240
75	1.9	Ductal/NST	0/7	51	Post	No	3
76	3.5	Mucoid	1/10	52	Post	No	62
79	1.8 (B)	Ductal/NST	3/15	33	Pre	No	7
81	-	Ductal/NST	18/19	64	Post	Yes	230
83	2.8	Ductal/NST	2/3	47	Pre	No	36
84	4.5	Ductal/NST	3/5	55	Post	No	0
85	5.5	Ductal/NST (M)	0/21	42	-	No	4
86	3.0	Ductal/NST (M)	0/21	42	-	No	51
87	2.5	Ductal/NST (M)	0/21	42	-	No	37
88	3.5	Ductal/NST	1/4	41	-	No	1
89	1.4 (B)	Lobular variant	0/4	39	Pre	No	35
90	1.4	Ductal/NST	1/1	56	Post	No	243
91	3.2	Ductal/NST	1/11	33	Pre	No	0
92	1.2	Ductal/NST	0/5	38	Peri	Yes	61
93	1.4	Ductal/NST	1/4	57	-	No	119
94	2.8	Ductal/NST	0/12	60	Post	No	268
95	2.0	Ductal/NST (M)	-	87	Post	No	11
96	1.6	Ductal/NST (M)	-	87	Post	No	-
97	1.3	Ductal/NST (M)	-	87	Post	No	-
98	3.8	Ductal/NST	0/12	75	Post	No	0
99	2.9	Ductal/NST	0/4	59	Post	No	277
100	3.8	Tubular	0/12	48	Pre	No	88
101	3.5	Comedo	0/12	59	Post	No	0
103	2.5	Papillary	6/12	68	Post	No	195
106	1.5	Ductal/NST	0/4	40	-	No	21
107	2.3	Tubular (M)	3/8	64	Post	Yes	149
108	1.7	Ductal/NST (M)	0/14	64	Post	Yes	321
109	2.1	Ductal/NST	0/3	56	Post	No	75
110	1.2	Ductal/NST	0/4	65	Post	Yes	570
112	1.8	Papillary	0/10	58	-	No	232
113	-	Medullary	0/4	35	-	No	9
114	1.4	Ductal/NST	0/12	36	Post	Yes	4

M/P - menopausal
ER - oestrogen receptor
NST - No Special Type
(B) - Biopsy
(M) - Several of the samples in the tumour bank are multiple primary breast tumours, independently occurring tumours from in the same patient (tumours 11 and 12; 14 and 15; 18 and 19; 68 and 69; 85, 86 and 87; 95, 96 and 97; 107 and 108). During this study a mutation in the p53 gene, CGA>TGA at codon 213, was identified in tumours 95, 96 and 97, strongly indicating a common origin for these three tumours. Consequently the three samples have been treated as a single tumour in the present study and are referred to collectively as tumour 95.

Summary of patient and breast tumour data:

Number of patients with primary breast cancer = 85
Number of primary breast tumours = 92

Number of tumours with information on:
Size = 67 (tumour sizes from biopsy samples have not been included)
Tumour type = 86
ER level = 84
Nodal status = 78

Number of patients with information on:
M/P status = 63
Age = 84
Family History = 85

Tumour Categorisation:

Tumour size	Nodal Status	ER level
<2cm = 23	Positive = 38	≤20pg = 27
2-5cm = 41	Negative = 40	>20pg = 57
>5cm = 3		

Tumour type:

Ductal/NST = 71	Tubular = 4
Lobular variants = 2	Mucoid = 2
Papillary = 2	Comedo = 1
Squamous = 1	Scirrhous = 1
Medullary = 1	Carcinoid = 1

Patient Categorisation:

Menopausal status	Age	Family History
Pre = 10	<50yrs = 23	No = 73
Peri = 5	≥50yrs = 61	Yes = 12
Post = 48		

2.2 Extraction of genomic DNA.

All chemicals unless otherwise stated were supplied by BDH Analar.

The extraction of genomic DNA followed the method of Gross - Bellard (1972). 10ml of lysis buffer (0.1M Tris HCL, 20mM NaCl, 1mM diaminoethanetetra - acetic acid (EDTA), 0.2% sodium dodecyl sulphate (SDS)) and 10ml of whole blood were mixed in a Falcon tube and incubated overnight at 4°C. 10ml of water-saturated phenol was added and after shaking the aqueous and phenol phases were separated by centrifugation at 3000rpm for 10 minutes. The aqueous layer was transferred to a fresh tube, 5ml of 7.5M ammonium acetate and 20ml of isopropyl alcohol were added and the solution was incubated overnight at -40°C. Precipitated DNA was spooled out on a plastic rod, air dried and resuspended in 10ml of resuspension buffer (10mM Tris HCl, 150mM NaCl, 10mM EDTA). The DNA was treated with 500µg of RNase (Sigma) for 30 minutes at 37°C followed by treatment with 100µg of proteinase K in the presence of 0.2% SDS for one hour at 37°C. 10ml of water-saturated phenol was added, the mixture shaken and incubated for 20 minutes on ice. The aqueous layer was recovered using centrifugation as before and two subsequent extraction steps were performed using 24:1 chloroform/isoamylalcohol in lieu of phenol. The DNA was ethanol precipitated from solution, spooled out and air dried as before. The remaining supernatant was centrifuged at 3000rpm for 15 minutes and any precipitated DNA was air dried in the tube. The DNA was suspended in 500µl of TE (10mM Tris HCL pH 7.5, 0.5mM EDTA) and stored at 4°C in screw cap Eppendorf tubes. After 4-7 days the DNA concentration was measured using a Pye Unicam SP-400 UV Spectrophotometer. An optical density of 1 at a wavelength of 260nm is equivalent to a DNA concentration of 50µg/ml.

DNA was extracted from approximately $1cm^3$ of solid tissue (breast tumour or placenta). Frozen tissue was minced finely into cubes of less than 1mm using a fresh single sided razor blade and transferred into 10ml of lysis buffer. From this point the tumour samples were treated as for whole blood. Lymphoblastoid cell lines were used as a renewable source of control DNA. Extraction of DNA from pellets containing $1x10^7$ cells was essentially the same as for whole blood. The cell pellet was resuspended in 10ml of lysis buffer and from then on treated as for whole blood.

2.3 Restriction enzyme digestion of DNA.

10µg of genomic DNA was digested with restriction enzyme in a 40µl or 60µl volume under conditions recommended by the manufacturers (Boehringer Mannheim, NBL, Bio Labs) plus 25mM spermidine (Sigma). Digestion reactions were normally left overnight at the appropriate temperature (37°C for all enzymes except TaqI which was incubated at 65°C). Following digestion 10µl of loading buffer (0.25% bromophenol blue (Sigma), 0.25% xylene cyanol FF (Sigma), 15% Ficoll (molecular weight 4 x 10^6) (Sigma) or 0.25% bromophenol blue, 40% sucrose) was added and the reaction mixture stored at 4°C until required. Details of enzymes used in this project are listed in table 2.2.

Table 2.2 Details of polymorphic DNA probes

Probe	Human Genome Mapping Locus	Map Location	Probe Size (kb)	Restriction Enzyme	Fragment Size (kb)
YNZ22	D17S5	17p13.3	1.7	TaqI	1.5 - 3.0
YNH37	D17S28	17p13.3	1.6	TaqI	2.0 - 4.0
c3068	-	17p13.2	8.8	HaeIII	1.0 - 4.0
MCT35	D17S31	17p13.1	2.4	MspI	1.8, 2.4
BHp53	TP53	17p13.1	4.1	BamHI	2.1, 4.1
THH59	D17S4	17q23 - q25.3	3.8	TaqI	0.8 - 1.8

2.4 Agarose gel electrophoresis.

Agarose gel electrophoresis is used to separate DNA molecules of different sizes. The passage of DNA through the gel is dependent on two major factors, the size of the DNA molecule and the concentration of the agarose gel. Agarose gel electrophoresis was used for three main purposes in this project. For the separation of restriction digested genomic DNA fragments (20 x 25cm) 500ml gels were used and were typically run at 60V for 18 hours. For the recovery of PCR amplified DNA fragments (10x15cm) 300ml gels were used and were typically run at 70V at 4°C for 2 hours. For the estimation of DNA concentration of recovered PCR amplified fragments 30ml gels (10 x 15cm) were used and were typically run at room temperature at 70V for 2 hours. The concentration of the gels ranged between 0.7% - 1% depending on the size of the DNA fragments being separated. Gels were made by heating a mixture of agarose (Sigma) and 1 x TAE (stored as 20

x TAE: 0.8M Tris, 0.4M sodium acetate, 20mM EDTA, pH 8.2 with glacial acetic acid) in a microwave oven until the agarose had melted. The solution was cooled to 60°C and poured into the appropriately sized gel tray. All agarose gels were run in 1 x TAE. DNA fragments were stained by immersing the gel in a solution of 0.5μg/ml ethidium bromide (Sigma) for 30 minutes followed by destaining with distilled water for 10 minutes. The DNA was visualised by placing the gel on a UV illuminator (UVP) and a record of the gel was taken either on Kodak X Pan 4147 film or by using a video camera (UVP) and copy processor (Mitsubishi).

2.5 Southern Blotting.

The protocol used for transferring DNA fragments onto a solid support broadly follows that of Southern (1975). Before blotting, gels were immersed in 1.25 litres of denaturing solution (0.5M NaOH, 1.5M NaCl) for 45 minutes followed by immersion in 1.25 litres of neutralising solution (2M NaCl, 1M Tris, pH 5.5) for 60 minutes. The blotting apparatus was prepared by placing a piece of glass (23cm x 48cm x 0.5cm) on a plastic tray (28 x 45cm) containing 1-2 litres of 20 x SSC (3M NaCl, 0.3M tri-sodium citrate). A 46 x 28cm piece of 17mm chromatography paper (Whatman), pre-soaked in 20 x SSC, was placed on the glass plate with both ends immersed in the 20 x SSC solution. Any air bubbles were removed by rolling a glass pipette over the paper. A piece of 23 x 25cm x 3mm chromatography paper (Whatman) was placed onto the 17mm paper wick, saturated with 20 x SSC and any air bubbles removed as before. The gel was removed from the neutralising solution and carefully placed on the 3mm paper to avoid air bubbles. The gel was cut through the loading wells with a single sided razor blade and the agarose strip discarded. Cling film was wrapped around the blotting apparatus in order to reduce evaporation from the 20 x SSC solution. A 23 x 20cm piece of Hybond N hybridisation membrane (Amersham) was placed on the gel and covered by two pieces of 3mm chromotography paper, a 0.5cm layer of white kitchen towel and a 7cm layer of green paper towel (Kimberly Clark). A plastic tray and 1kg weight were placed on top of the towels and the assembly was left overnight. The towel and filter paper layers were removed and the Hybond filter marked with a name, the date and the position of the wells with a ball point pen. The top right hand corner of the filter was cut in order to determine orientation. The filter was

removed from the gel, placed DNA side down on a clean UV transilluminator and illuminated for 4 minutes in order to fix the DNA to the Hybond membrane. It was then placed between two sheets of 3mm chromotography paper and baked for 60 minutes in an 80°C vacuum oven. The gel was removed from the blotting apparatus, stained with ethidium bromide and checked on a UV transilluminator for efficient transfer of DNA onto the Hybond membrane. Alternatively, before blotting, the gel was soaked for 30 minutes in denaturing solution and blotted for 4 hours onto Hybond N+ hybridisation membrane (Amersham) using denaturing solution in lieu of 20 x SSC. After blotting the hybond N+ filter was rinsed in 2 x SSC and air dried.

2.6 Radiolabelling of probe DNA.

All probes were supplied by Dr Y. Nakamura, Department of Biochemistry, Cancer Institute, Tokyo 170, Japan. (Listed in Table 2.2). All probes were grown by Mrs Irene Mackenzie using standard techniques (Sambrook *et al.*, 1989). Probes used for Southern hybridisation were labelled with ^{32}P dCTP (Amersham) using random priming kits (Amersham). Routinely 25ng of probe DNA was denatured in 10µl of distilled water by heating to 100°C for 10 minutes and immediately cooling on ice. 2µl of random prime buffer, 1µl each of 0.5mM dATP, 0.5mM dGTP and 0.5mM dTTP solutions, 5µl of ^{32}P dCTP and 1µl (2 units) of the Klenow fragment of DNA polymerase 1 were added. The reaction mixture was incubated at 37°C for 1 hour. The incorporation of ^{32}P dCTP was checked by pipetting 0.5µl of the mixture onto a circle of Whatman GF/B filter paper and comparing the radioactive counts before and after 3 x 10 ml washes of 5% trichloroacetic acid. If incorporation of over 40% was achieved then the probe was precipitated by adding 1µl of 3M sodium acetate and 140µl of ethanol and cooling at -40°C for an hour. The probe was recovered by centrifugation at 13000 rpm for 30 minutes at 4°C, resuspended in 100µl of 10mg/ml denatured salmon sperm DNA (Sigma), heated to 100°C for 10 minutes and added to the prehybridised filter. When using the probes MCT35.1 and c3068, which contain human DNA repeat sequences, 100µl of 1mg/ml denatured sonicated human placental DNA was added in addition to the denatured salmon sperm DNA. After heating at 100°C for 10 minutes these probes were incubated at 65°C for 60 minutes.

2.7 Nucleic acid hybridisation.

Both prehybridisation and hybridisation of Hybond filters were carried out in quick-hybe hybridisation mix (250ml 20 x SSC, 0.5g Bovine Serum Albumin (Sigma), 0.5g polyvinylpyrrolidine (Sigma), 0.5g Ficoll, 1g SDS, 1g sodium pyrophosphate (Sigma), 5ml of 20mg/ml denatured salmon sperm DNA, made up to 1 litre of solution). Filters were sealed in a plastic bag with about 1ml of quick-hybe per $15cm^2$ of filter, ie typically 30ml of quick-hybe mix for a 23 x 20cm Hybond filter. The filters were prehybridised for at least one hour in a 65°C waterbath. The radioactively labelled probe was then added to the hybridisation mixture and sealed, ensuring no air bubbles were retained in the bag. The filter was hybridised overnight at 65°C. The hybridisation bag was opened carefully and the contents emptied under 500ml of 2 x SSC, 0.1% SDS wash solution. The wash solution was discarded and the filter washed twice using 500ml of fresh 2 x SSC, 0.1% SDS wash solution and once using either 500ml of 0.1 x SSC, 0.1% SDS wash solution or 0.2x SSC, 0.1% SDS wash solution (depending on the probe) in a shaking oven at 65°C for 10 minutes. All wash solutions were preheated to 65°C before use. After washing, the filters were wrapped in clingfilm, placed in an X-ray cassette and autoradiographed at -70°C overnight with Kodak X-AR Omat film. Film was processed in a Fuji RGB-2 Automatic film processor.

Alternatively a Hybaid hybridisation system was used which replaces hybridisation bags with bottles. After blotting and fixation of DNA the filter was placed between two layers of nylon mesh and soaked in 2 x SSC. The filter/mesh sandwich was rolled up, placed in a glass screw topped hybridisation bottle with 200ml of 2 x SSC and carefully unrolled ensuring no air bubbles were trapped between the filter and the bottle. The 2 x SSC was discarded and replaced by 20ml of quick-hybe. Prehybridisation, hybridisation and washing steps as described above were carried out in the hybridisation bottle at 65°C within a specially designed rotary incubator.

2.8 PCR amplification of genomic DNA.

The oligonucleotide primers used in this project were synthesised by an Applied Biosystems 391 oligonucleotide synthesiser. Oligonucleotide stocks were stored in 30% ammonium hydroxide at -20°C. Oligonucleotide primers were precipitated from the stock solution before use by adding 35µl

of 3M sodium acetate pH 5.5 and 770µl of ethanol to 350µl of stock solution and cooling for 60 minutes at -20°C. The solution was centrifuged at 13000 rpm for 15 minutes at 4°C and the supernatant discarded. The pellet was washed twice with 80% ethanol, air dried and resuspended in 200µl TE. The concentration of the primers was calculated by optical density reading (section 2.2). The molarity of the primers was calculated using the following method.

The exact extinction coefficient of a particular oligonucleotide (E) can be calculated by adding the extinction coefficient for each base. Extintion coefficients (ml/mole) for each base are: dATP 15.4, dCTP 7.3, dGTP 11.7, dTTP 8.8.

The concentration of the primer is calculated from the formula:

Concentration in µM = Optical density at 260nM/E.

The protocol for PCR amplification of genomic DNA is that of Saiki *et al.*, 1988. PCR reactions were carried out in a 100µl reaction volume using DNA primers at 5 µM, dNTPs (dATP, dCTP, dGTP, dTTP) at 20mM, 0.5-1µg of genomic DNA, 3 units of Promega Taq polymerase enzyme and Promega Taq reaction buffer as recommended by the manufacturer. The DNA was amplified in a Perkin Elmer Cetus thermal cycler (Model 2.2) using the cycles of 94°C for 2 minutes, 55°C for 2 minutes, 72°C for 3 minutes for 30 cycles. The sequence of the oligomers were as follows:

p53 gene fragment III (exons 5 and 6, intron 5)

Primer III - 1. 5' TTCCTCTTCCTGCAGTACTC 3'

Primer III - 2. 5' AGTTGCAAACCAGACCTCAG 3'

p53 gene fragment IV (exons 7, 8 and 9, introns 7 and 8)

Primer IV - 1. 5' CTGTGTTATCTCCTAGGTTGG 3'

Primer IV - 2. 5' CCCAAGACTTAGTACCTGAA 3'

The amplified DNA fragments were recovered by running the reaction mixture out in a 1% low melting point agarose (Sigma) gel as detailed in section 2.4. After ethidium bromide staining the amplified DNA fragments were excised from the gel in an agarose slice using a single sided razor blade and placed into labelled Eppendorf tubes.

2.9 Purification of PCR amplified DNA fragments.

The geneclean kit (Bio 101 Inc) enables the purification of DNA molecules greater than 300bp in size from solution or agarose and was used to recover amplified DNA fragments from low melting point agarose gel slices. Genecleaning was carried out according to the manufacturers instructions. The volume of the gel slice was estimated by weight (1mg equals 1ml) and three times the volume of NaI stock solution was added to the slice. The mixture was incubated at 50°C for three minutes to ensure the agarose had completely melted. 5µl of glassmilk (silica bead solution) was added and, after vortexing, incubated on ice with occasional shaking for 20 minutes. The glassmilk beads were pelleted by centrifugation at 2500rpm for 7 seconds and the supernatant discarded. The pellet was cleaned three times by vortexing in 200µl of "New wash" solution and recovered by centrifugation as before. The glassmilk beads were suspended in 10µl of TE and incubated at 50°C for 3 minutes to ensure dissociation of the DNA from the beads. The supernatant was removed from the beads after centrifugation for 30 seconds. This eluting step was carried out once more with a separate 10µl of TE to ensure maximum recovery of DNA. The concentration of the DNA in solution was estimated by running 3µl out on a 1% agarose gel along with 1µl of 1mg/ml HindII/EcoR1 cut lambda DNA (Boehringer Mannheim) as detailed in section 2.4.

2.10 Kinase labelling of double stranded PCR amplified products.

End labelling of DNA fragments was achieved by the use of the enzyme T4 polynucleotide kinase which transfers the γ phosphate from ^{32}P γ ATP to the 5' OH group of a dephosphorylated oligonucleotide. 100ng of PCR amplified DNA was labelled in a reaction volume of 20µl containing 2µl of ^{32}P γ ATP, 2µl of 10 x kinase buffer and 1µl (9units) of T4 polynucleotide kinase. The mixture was incubated at 37°C for 30 minutes. The DNA was precipitated by adding 20µl of TE, 5µl precipitating salts (3M sodium acetate pH 7.5, 7.5M ammonium acetate), 1.5µl of 1mg/ml glycogen (Boehringer) and 150µl of ethanol and incubated for half an hour at -70°C. The DNA was pelleted by centrifugation at 13000 rpm at 4°C and then washed twice in 70% ethanol to remove unincorporated nucleotides. The resulting pellet of end labelled DNA was dried under vacuum and resuspended in 10µl of TE.

2.11 DNA heteroduplex formation.

10ng of labelled probe DNA was mixed with 100ng of cold target DNA in a reaction volume of 10µl containing 1µl of 10 x heteroduplex salts (1M Tris, 3M NaCl, pH 8.0) and overlaid with mineral oil (Sigma). The mixture was heated to 100°C for 5 minutes, transferred to a 65°C waterbath and left overnight. The DNA, including the reannealed heteroduplex molecules, was precipitated by adding 1.5µl 10mg/ml glycogen, 5µl precipitating salts and 150µl of ethanol followed by incubation for 30 minutes at -70°C. The DNA was recovered by centrifugation for 15 minutes at 4°C, washed twice in 70% ethanol and dried for 60 seconds in a vacuum chamber. The resulting pellet was resuspended in 13 µl TE. 6µl was transferred into a new tube and treated with osmium tetroxide (Aldrich or Johnson Matthey), the remaining 7µl was treated with hydroxylamine hydroxide (Aldrich).

2.12 Chemical modification of mismatches using hydroxylamine hydroxide and piperidine.

20µl of a fresh 4M solution of hydroxylamine hydroxide (HA) titrated to pH 6 with diethylamine (Aldrich) was added to the 7µl of heteroduplex solution and incubated at 30°C for 2 hours. The reaction was stopped by adding 200µl of 0.3M sodium acetate, 0.1mM EDTA solution, 5µl of 10mg/ml dextran and 750µl of ethanol. The DNA was incubated for 10 minutes in a methanol/dry ice bath, recovered by centrifugation at 13000rpm for 15 minutes at 4°C, washed twice with 70% ethanol and dried for 60 seconds in a vacuum chamber. To the pellet 50µl of fresh 1M piperidine (Aldrich) solution was added, vortexed for at least 60 seconds, and incubated at 90°C for 30 minutes. The reaction was stopped and precipitated as before but without dextran. The pellet was resuspended in 5µl of TE and 2µl of stop solution (95% formamide, 20nM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol FF) added. Immediately prior to denaturing polyacrylamide electrophoresis the mixture was heated at 100°C for 2 minutes and stored on ice.

2.13 Chemical modification and cleavage of mismatches using osmium tetroxide and piperidine.

15µl of a fresh 0.8% (1/10 dilution of a 8% solution or a 1/5 dilution of a 4% solution) osmium tetroxide solution (OsO_4) (Aldrich or Johnson Matthey) and 2.5µl of osmium buffer

(100mM Tris pH7.7, 10mM EDTA, 15% pyridine (Aldrich)) were added to the 6µl of heteroduplex solution. The solution was mixed (but not spun down) and incubated for 37°C for 30 minutes. The reaction was stopped by adding 200µl of 0.3M sodium acetate, 0.1mM EDTA solution, 5µl dextran and 750µl of ethanol and from this step, cleaved with piperidine in the same way as for HA modification.

2.14 Sequencing of double stranded DNA amplified PCR fragments.

The sequencing of dsDNA PCR fragments was carried out using the Sequenase DNA Sequencing Kit (USB) essentially following a modified protocol of Winship (1989). 50ng of template DNA, 200 - 250ng of DNA oligonucleotide primer, 1µl of dimethyl sulphoxide (DMSO) and 2µl of sequenase reaction buffer in a volume of 10µl was heated at 100°C for 2 minutes and immediately transferred to a dry ice/methanol water bath. 1µl of dithiothreitol (DTT), 0.5µl ^{32}P dCTP, 2µl label mix (1.5µM dATP, 1.5µM dGTP, 1.5µM dTTP) and 2 units of sequenase in 1.5µl of sequenase enzyme dilution buffer (10mM Tris-HCl pH 7.5, 5mM DTT, 0.5mg/ml BSA) were added, the solution mixed and incubated for 5 minutes at room temperature. 3.2µl of the mixture was aliquoted into four tubes each containing 2µl of 80µM dATP, 80µM dCTP, 80µM dGTP, 80µM dTTP, 50mM NaCl and either 8µM ddATP (tube A), 8µM ddCTP (tube C), 8µM ddGTP (tube G) or 8µM ddTTP (tube T). The tubes were incubated for 5 - 10 minutes at 37°C and the reaction stopped by adding 4µl of stop solution. Immediately prior to denaturing polyacrylamide gel electrophoresis the mixture was heated to 100°C for 2 minutes and transferred to ice.

The DNA primers used for PCR amplification (section 2.8) were used as primers for sequencing. In addition the following primers were used:

p53 gene fragment III (for sequencing exon 6)

III - 3. 5' GACCTCAGGCGGCTCATA 3'

p53 gene fragment III (for sequencing exon 6)

III - 4. 5' CCTCTGATTCCTCACTGA 3'

p53 gene fragment IV (for sequencing exon 9)

IV - 3. 5' TAGTACCTGAAGGGTGAA 3'

p53 gene fragment IV (for sequencing exon 8)

IV - 2A. 5' GTCCTGCTTGCTTACCTCGCT 3'

2.15 Denaturing polyacrylamide gel electrophoresis.

Denaturing polyacrylamide gel electrophoresis was carried out using the Bio rad sequi-gen sequencing apparatus according to the manufacturers instructions. A 6% acrylamide/urea solution was used for all polyacrylamide gel electrophoresis. 57g acrylamide (BRC), 3g N, N' - methylenebisacrylamide (BDH) and 460g urea were dissolved at 37°C in 900ml of distilled water and treated with 20g of 'Amberlite' MB-1 monobed mixed resin for 30 mins. 50mls of 20 x TBE (1.8M Tris, 1.8M Boric acid, 0.04M EDTA) was added and the final volume made up to 1 litre. The gels were made and run between two glass plates, the large plate containing the electrodes and buffer tank, separated by two 0.4mm plastic spacers. The surface of the large plate was coated with 2% dimethyldichlorosilane in trichloroethane to ensure that the polyacrylamide gel adhered to the small plate only. The large and small plates were rinsed successively with tap water, distilled water and ethanol to ensure a clean surface. The two gel plates were held together by plastic clamps and once in place the plug of the gel was poured. The casting tray contained a strip of 23.5 x 3cm 17mm chromatography paper resting on a 23.5cm x 3cm foam pad. 40µl of N, N, N', N' - tetramethylethylenediamine (TEMED) (Sigma) and 240µl of 10% ammonium perisulphate (AMPS) (BRL) were added to 15ml of 6% acrylamide/urea solution and immediately poured into the casting tray. The assembled gel apparatus was placed into the casting tray and secured using plastic screws. Once the plug was set 380µl of AMPS and 55µl of Temed was added to 45ml of 6% acrylamide/urea solution and poured between the two gel plates using a 50ml syringe. The appropriate comb was inserted (a 0.4mm, 48 well Sharkstooth comb for sequencing gels or a 0.4mm, 16 well N-comb for HOT gels (Bio - Rad)) and the gel allowed to set for 60 minutes. After setting, any excess acrylamide was removed, the gel placed into the base apparatus and the tray and buffer tank filled with 400ml and 450ml of 1 x TBE respectively. The gel was run at 30 Watts for 15 minutes and the comb carefully removed. The wells were rinsed well with 1 x TBE buffer to remove urea before loading the samples and, once loaded, the gel was run at 30 watts for up to 6 hours depending on the

size of the fragments to be resolved. After electrophoresis the gel was fixed for 10 - 15 minutes in a 10% methanol, 10% acetic acid solution, transferred to 3mm chromatography paper, covered with cling film and dried at 80°C on a vacuum gel drier (Bio - Rad). After drying the cling film was removed, the gel placed in an X-ray cassette and autoradiographed at -70°C overnight with Kodak X-AR 5 film. Subsequent exposures were carried out according to the strength of the signal.

Chapter 3

Loss of heterozygosity on chromosome 17 in primary human breast cancer

3.1 LOH in human breast cancer.

LOH in breast tumours has been studied by many independent groups and the criteria by which a tumour is classed as having 'lost' or 'not lost' heterozygosity at a particular locus vary widely. Breast tumour samples often show a less than total loss of an allele due to either tumour heterogeneity or contamination of the tumour material with normal tissue and are classed as showing partial LOH. Recent LOH studies, discussed below, confirm that many breast tumour samples contain more than one clone or are heavily contaminated with normal tissue.

3.2 Composition of breast tumour samples.

Unlike other solid tumours there are intrinsic difficulties associated with the study of LOH in breast cancer. Removal of normal cells from tumour samples has been achieved in cancer of the colon (Vogelstein *et al.*, 1989), lung (Rabbitts *et al.*, 1989), oesophagus (Boynton *et al.*, 1991), brain (Cogen *et al.*, 1990) and pancreas (Hohne *et al.*, 1992). However breast tumour samples are very frequently contaminated with normal tissue in such a way that the separation of malignant and non-malignant cells is not feasible. The extent of this contamination in breast tumour samples has been examined by several groups by histological analysis. Almost half of the samples in the tumour series used by Devilee *et al.* (1989) contained less than 60% malignant cells. The malignant cell content ranged from 30-100% and 20-100% in two tumour series studied by Larsson *et al.* (1990) and Thorlacius *et al.* (1991). Varley *et al.* (1989) reported that less than half the cells in the majority of tumour samples were malignant and between 75-90% of cells were identified as malignant in tumour samples used by Lundberg *et al.* (1987). From these reports it is clear that the composition of breast tumour samples vary widely between different tumour banks.

3.3 Heterogeneity in breast tumour samples.

Breast cancer is a very heterogeneous disease in terms of tumour characteristics, disease course and treatment response and this is reflected in findings at the molecular level. In addition to the variation found between breast tumours, termed 'intertumour variation', cellular variation within breast tumours, termed 'intratumour variation', has also been observed (Wolman and Heppner 1992).

Although breast tumours are thought to arise from a single cell, various sublines can develop during the evolution of the tumour which differ in genetic characteristics (Heppner 1984). Tumour heterogeneity, the term given to the occurrence of more than one type of cell in a single tumour, has been well documented in breast cancer at cytogenetic and cytometry levels (Devilee and Cornelisse 1990). The extent of tumour heterogeneity in breast tumour samples has also been determined in two studies at the LOH level (Larsson *et al.*, 1990; Chen *et al.*, 1992).

The study by Chen *et al.* (1992) identified tumour samples which showed complete allele loss at one locus and a partial allele loss at a second site. Complete loss of an allele in these samples indicated that the amount of contamination by normal tissue was very small. The partial loss indicated that only a subset of the cells in the tumour actually showed LOH at the second site, suggesting more than one clone of cells existed within the tumour. In 34 cases where complete LOH was observed a partial LOH was observed at a second site in 8 tumours (24%). Since only twelve sites on 10 chromosomes were investigated the extent of partial LOH in this tumour bank is likely to be an underestimate.

In the study by Larsson *et al.* (1990) the percentage of malignant cells in the tumour material had been determined by histological analysis. Thus any reduction in allele intensity could be predicted in a tumour sample showing LOH. A decrease in the expected figure was attributed to the tumour containing one or more clones which did not show LOH. This tumour heterogeneity was observed in the majority (68%) of the tumours analysed. Clearly both these reports suggest that breast tumour samples frequently contain more than one type of clone.

3.4 Criteria for LOH status in breast cancer.

Variation in allele intensity detected between blood and tumour DNA by Southern blot analysis is thought to be due in part to experimental variation. Due to this variation, tumours showing only partial loss of an allele are separated into groups showing 'insignificant' and 'significant' loss. Setting a level of significance has been achieved in a number of ways.

Several groups have relied on a visual evaluation of the relative allele ratios (Ali *et al.*, 1987; Mackay *et al.*, 1988a; Mackay *et al.*, 1988b; Ali *et al.*, 1989b; Coles *et al.*, 1990; Cropp *et al.*,

1990; Gendler *et al.*, 1990; Sato *et al.*, 1990; Thompson *et al.*, 1990; Leone *et al.*, 1991; Sato *et al.*, 1991a; Thorlacius *et al.*, 1991; Varley *et al.*, 1991; Bieche *et al.*, 1992; Borg *et al.*, 1992b,c; Cheickhi *et al.*, 1992; Takita *et al.*, 1992; Thompson *et al.*, 1992) but this approach is highly inaccurate unless tumour samples with very small amounts of normal tissue contamination and low tumour heterogeneity are analysed. Other investigators have quantitatively determined the signal imbalance by use of a scanning densitometer (Chen *et al.*, 1989; Devilee *et al.*, 1989; Genuardi *et al.*, 1989; Borresen *et al.*, 1990; 1992; Andersen *et al.*, 1992; Kallioniemi *et al.*, 1992; Matsumura *et al.*, 1992; Knyazev *et al.*, 1993) and then set an arbitrary value of significance. Relative signal reduction values used to classify the 'no loss' and 'loss' states in breast tumours include 30% (Chen *et al.*, 1992; Kallioniemi *et al.*, 1992; Matsumura *et al.*, 1992), 35% (Chen *et al.*, 1989), 40% (Devilee *et al.*, 1989) and 50% (Andersen *et al.*, 1992; Knyazev *et al.*, 1993).

Both these approaches are unsatisfactory since they do not take into account the possibility of normal cell contamination or intratumour heterogeneity. Both these variables effect the reduction of allele intensity observed in the southern blot assay and so can effect the estimation of the frequency of LOH at a particular locus. Tumour samples highly contaminated with normal tissue or containing genetically different subclones can lead to an underestimation of the frequency of LOH when using the above criteria. Methods which take both of these factors into account have been devised by Lundberg *et al.* (1987), Devilee *et al.* (1990) and Larsson *et al.* (1990). All the tumours analysed for LOH in these studies had the amount of non-malignant tissue determined by scoring representative histological sections at low magnification. Thus it was possible to predict the expected imbalance in allele ratios in each tumour assuming all the malignant cells show LOH. Any reduction in this estimated figure was attributed to tumour heterogeneity.

3.5 Determining LOH on chromosome 17 in primary breast tumours.

This series of experiments followed on from the work of Mackay *et al.* (1988a) investigating the high frequency of LOH observed in primary breast tumour samples on chromosome 17. LOH was identified in 23/38 (61%) of samples in the breast tumour bank using the VNTR probe YNZ22 (Mackay *et al.*, 1988a). The present set of experiments determined the rates of LOH at other loci on

chromosome 17 and had a twofold objective. Firstly LOH information on loci mapping adjacent to the YNZ22/D17S5 locus was needed in the hope of identifying a SRO pinpointing an area of the chromosome likely to harbour a putative tumour suppressor gene. Secondly the LOH frequency at loci mapping close to the p53 gene itself could be used to investigate a potential role for p53 in breast carcinogenesis.

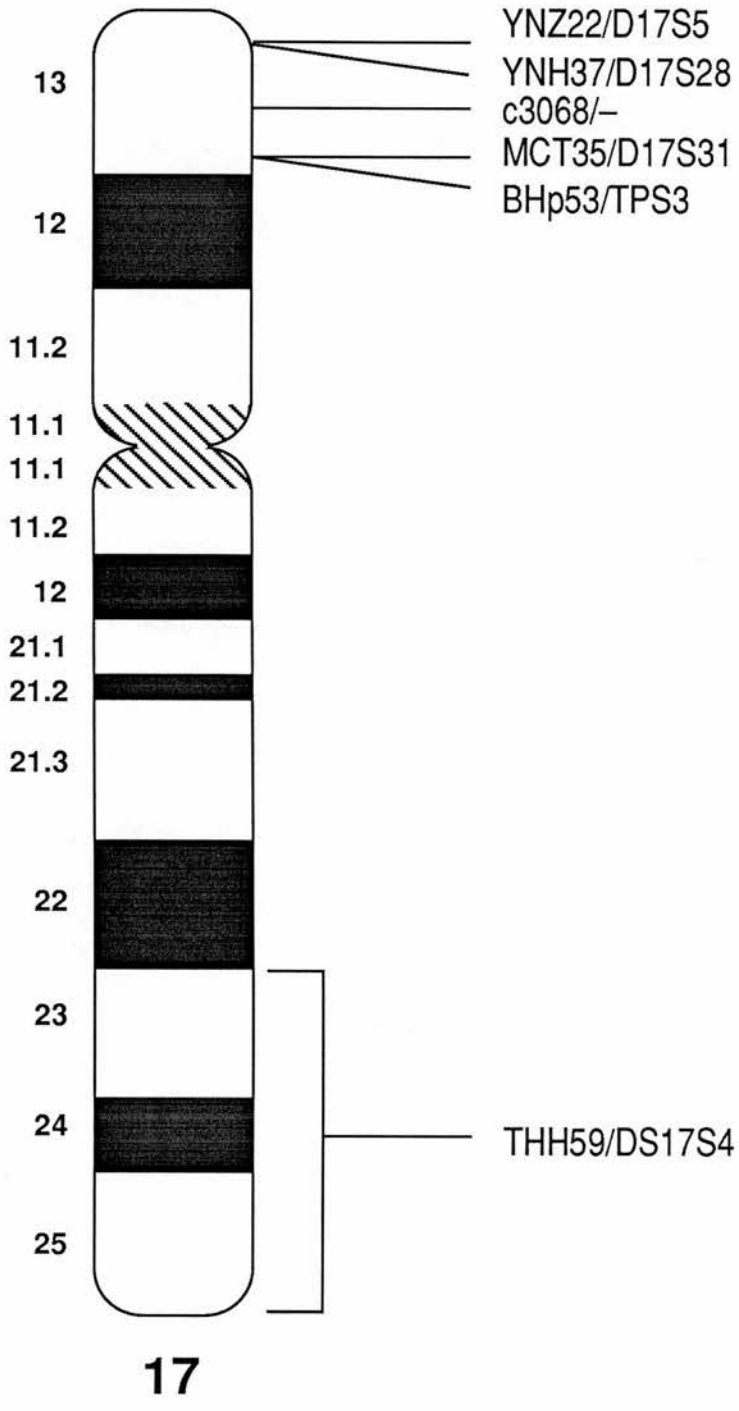
The work was carried out on the bank of paired control/tumour DNA samples established by Mackay *et al.* and detailed in section 2.1. Previous investigation of LOH at various loci had depleted the DNA bank of certain tumour and blood DNA samples (Mackay *et al.*, 1988a,b). Of the 92 samples of matched control/primary breast cancer DNA only 61 pairs were found to contain enough DNA for LOH analysis. Using these samples the following probes were used to determine LOH: BHp53, MCT35, c3068, THH59 and YNH37, which map to 17p13.1, 17p13.1, 17p13.2, 17q23-25.3 and 17p13.3 respectively (Figure 3.1). The frequency of heterozygosity observed at these loci is shown in table 3.1.

3.6 Measurement of reduction in allele intensity.

Autoradiographs were analysed using the MRC HGU large format densitometer and tracks unable to be analysed due to high background levels were not included in the study. Reduction in the ratio of allele intensities was calculated after equalising one of the control alleles with the corresponding tumour allele. The intensity of the two remaining alleles could then be compared directly and any reduction in signal calculated. Figure 3.2 shows representative examples of southern blot analysis in various control/tumour DNA pairs using a variety of probes. Figure 3.3 shows the densitometer scan figures used in calculating the relative signal reduction in tumour 99 at the c3068/- locus.

In total 79 loci in 40 tumours were measured quantitatively by scanning densitometry. After adjusting for background levels, the relative reduction of allele intensities were calculated and these figures are shown in table 3.2. Since the LOH data on the YNZ22/D17S5 locus (Mackay *et al.*, 1988a) had only been analysed visually it would have been of value to re-analyse the original autoradiographs by densitometry in order to be able to compare them directly with the data from

Figure 3.1



Location of the loci on chromosome 17 identified by the probes used in this study.

Figure 3.2 Southern blot analysis of pairs of constitutional (N) and tumour (T) DNA using six different chromosome 17 probes. The relative reduction in allele intensity is shown as a percentage.

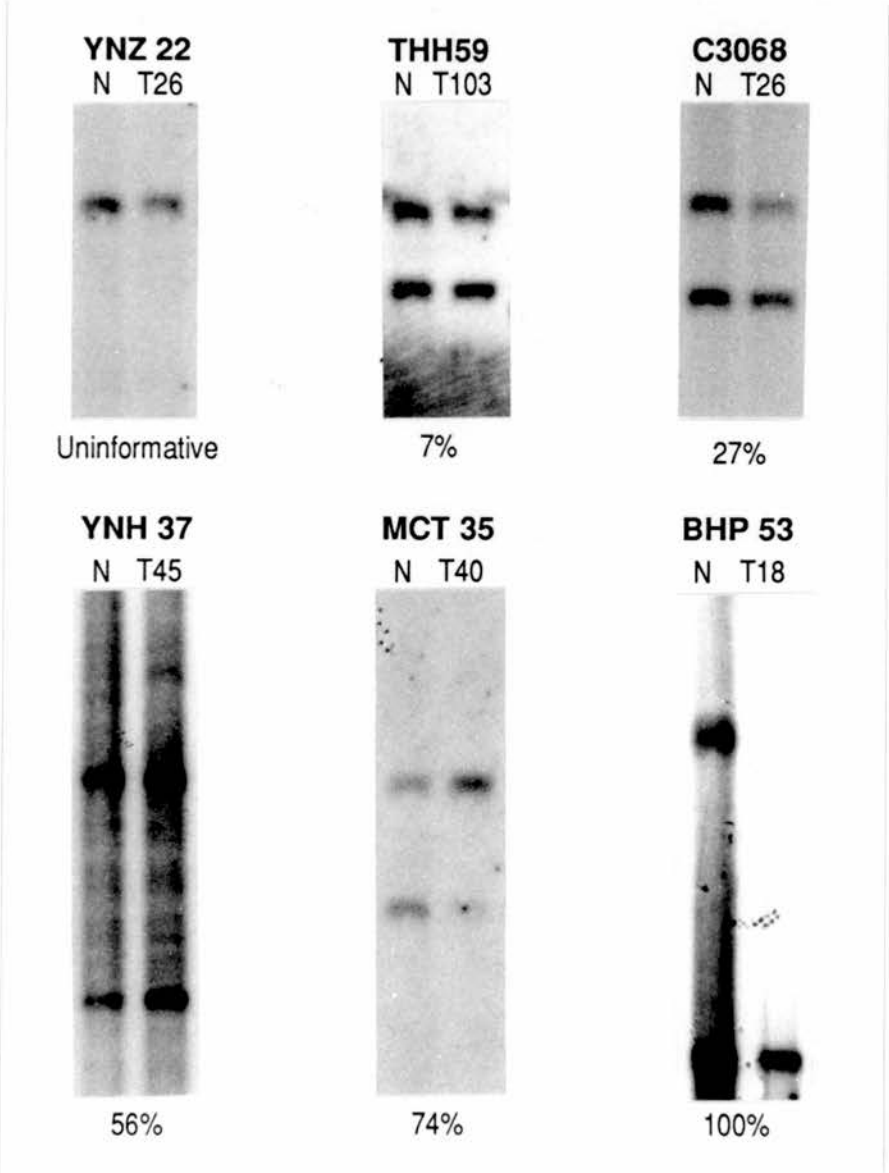
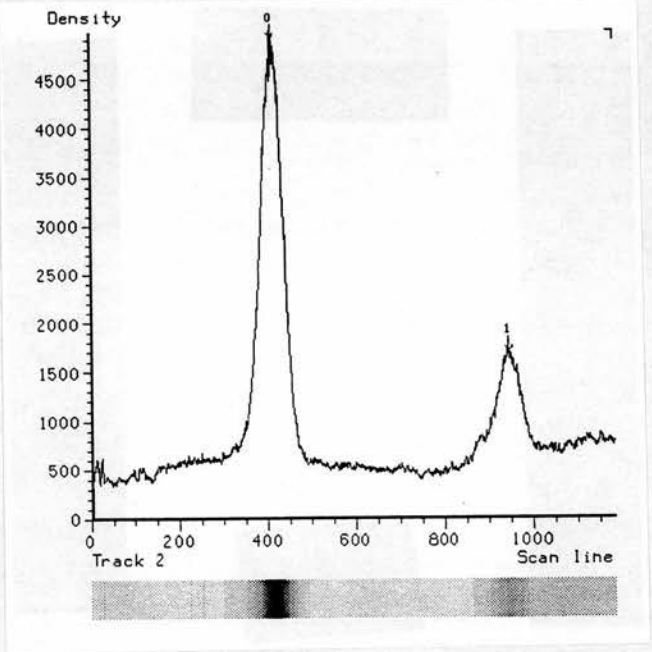
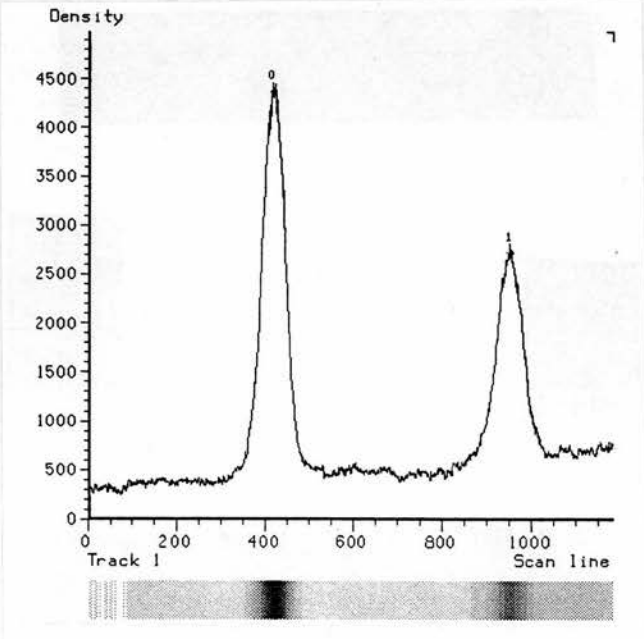


Figure 3.3 Densitometer scans of an autoradiogram obtained following hybridisation of c3068 to an HaeIII digest of tumour 99 DNA (A) and corresponding normal DNA (B).

A



B



other loci on chromosome 17. Unfortunately reanalysis of these autoradiographs was not possible. Due to the decreasing amount of DNA available for LOH studies and experimental difficulty with the YNZ22 probe only 7 informative control/tumour pairs were analysed at the YNZ22/D17S5 locus. These results and the visual evaluation of the control/tumour pairs by Mackay *et al.* are shown in table 3.2.

Table 3.1 Frequency of heterozygosity detected at chromosome 17 loci in primary breast cancer patients.

	Probe name/Locus name				
	THH59/ DS17S4	BHp53/ TP53	MCT35/ D17S31	c3068/-	YNH37/ D17S28
Number of breast cancer patients	13	44	31	23	12
Number of heterozygous patients	6	22	16	14	8
Frequency of heterozygosity (%)	46	50	51	61	67

3.7 Analysis of quantitative values.

The percentage of malignant cells in the breast tumour samples had not been determined prior to DNA extraction. Therefore, as with other studies, a level of significance was set to provide an arbitrary cut off point of significance. Table 3.3 shows analysis of the data using 40% as a measure of significance for LOH.

A high frequency of LOH using the probe YNZ22 has been observed in many other studies (Mackay *et al.*, 1988a; Devilee *et al.*, 1989; Varley *et al.*, 1989; Borresen *et al.*, 1990; Cropp *et al.*, 1990; Larsson *et al.*, 1990; Sato *et al.*, 1990; Thompson *et al.*, 1990; Chen *et al.*, 1991a; Devilee *et al.*, 1991a; Osborne *et al.*, 1991; Thorlacius *et al.*, 1991; Andersen *et al.*, 1992; Chen *et al.*, 1992; Cheickhi *et al.*, 1992; Matsumura *et al.*, 1992; Cornelise *et al.*, 1993; Knyazev *et al.*, 1993). This frequency ranges from 17% (22/130) (Cheickhi *et al.*, 1992) to 69% (51/74) (Varley *et al.*, 1989). However LOH at this locus was observed in only 14% (1/7) of informative samples in this study (Table 3.3) and is probably a consequence of the small number of samples analysed. Other studies

Table 3.2 Loss of heterozygosity at chromosome 17 loci in primary breast tumours expressed as a relative percentage reduction of signal.

Tumour Number	TTH59/ D17S4	BHp53/ TP53	MCT35/ D17S31	c3068/-	YNH37/ D17S28	YNZ22 /D17S5	YNZ22 data Mackay *et al.*
1			U	U			NL
3			U				L
13				U			
14		36	43	28			L
15		45	65	64			L
16		52	69				U
18	U	100	U	U			L
19	U	23	U	U			NL
20	2	30	U	49			U
21		100	100	U	U		L
22		U	U				L
24	U	29		U	37	24	NL
26	21	U	30	27	22	U	L
28		U					NL
29		35					
30		U					
31							L
33		U					NL
34	U	U	70			25	L
35		50		37			NL
37		U	3				NL
38		U					
39					2		
40	72	71	76	69		U	U
42		42					
45	30	29	U	46	56		L
47				U			U
48			U				
50	U	U	U	28	47		U
52							NL
57			90				
58			U				U
59		U	75	U			
60		U					
63		7	18	40			L
64							L
65		13	4	19	U	16	NL
67		U					L
68		U	U				L
69		U	U				
70		U					
72		61			89		
74		36					
75		28					L
76						4	L
79		U	46	32			L

Table 3.2 (continued)

Tumour Number	TTH59/ D17S4	BHp53/ TP53	MCT35/ D17S31	c3068/-	YNH37/ D17S28	YNZ22 /D17S5	YNZ22 Data Mackay *et al.*
81		U					NL
85		U	16				U
86		U	29				U
87		U					U
90		U	36				
91		89	U				NL
92						78	
93	U	20		46			NL
94	U	U		68			NL
95	U	U	U				L
96							L
97							L
98			U				
99	42	58	U	58	58		U
100		U	82	U	U	5	NL
101		100					L
102							NL
103	7	26	22	8	10	16	NL
107		34		U	U	U	U
108		29		U	U	U	U
109		U	U				

U - Uninformative
NL - No LOH
L - LOH

have based their figures on sample sizes ranging from 19 (Osborne *et al.*, 1991) to 130 (Cheickhi *et al.*, 1992) informative samples. In this study LOH is observed in 50% (4/8) of informative samples at the YNH37/D17S28 locus (Table 3.3), which maps only 20 kb from the YNZ22/D17S5 locus (Ledbetter *et al.*, 1989). Although this frequency approaches those observed for the YNZ22/D17S5 loci in other studies an accurate estimation was not possible as only eight informative samples were available for analysis.

Table 3.3 Frequency of LOH at loci on chromosome 17

	Probe name/Locus name					
	THH59/ D17S4	BHp53/ TP53	MCT35/ D17S31	c3068/-	YNH37/ D17S28	YNZ22/ D17S5
Number of informative samples	6	25	18	15	8	7
Number of samples showing LOH	2	11	10	8	4	1
Frequency of LOH (%)	33	44	56	53	50	14

LOH at loci near the p53 locus, as defined by the probes BHp53 and MCT35, was observed in 17/35 (49%) of informative samples. This figure falls within the range observed in other studies: 61% (17/28) (Davidoff *et al.*, 1991a); 37% (26/70) (Sato *et al.*, 1991a); 30% (6/19) (Osborne *et al.*, 1991); and 48% (19/42) (Andersen *et al.*, 1992). LOH at the THH59/D17 locus on the long arm of chromosome 17 was shown in 2/6 (17%) of informative samples. This frequency is less than that found at this locus by Cropp *et al.*, (1991) (29%) although, as with the YNZ22/D17S5 and YNH37/D17S28 data, the sample size is too small to produce an accurate estimate of LOH.

While studies show a similar range of LOH frequencies for both the YNZ22/D17S5 and BHp53/TP53 loci three studies investigating both loci show a marked difference in frequency of LOH (Coles *et al.*, 1990; Sato *et al.*, 1990; Andersen *et al.*, 1992). In the study by Sato *et al.* (1990) loci at 17p13.3 showed LOH at a higher frequency (52%) than those at 17p13.1 (32%). In the study by Andersen *et al.* (1992) loci mapping to 17p13.3 were shown to have a frequency of LOH as high

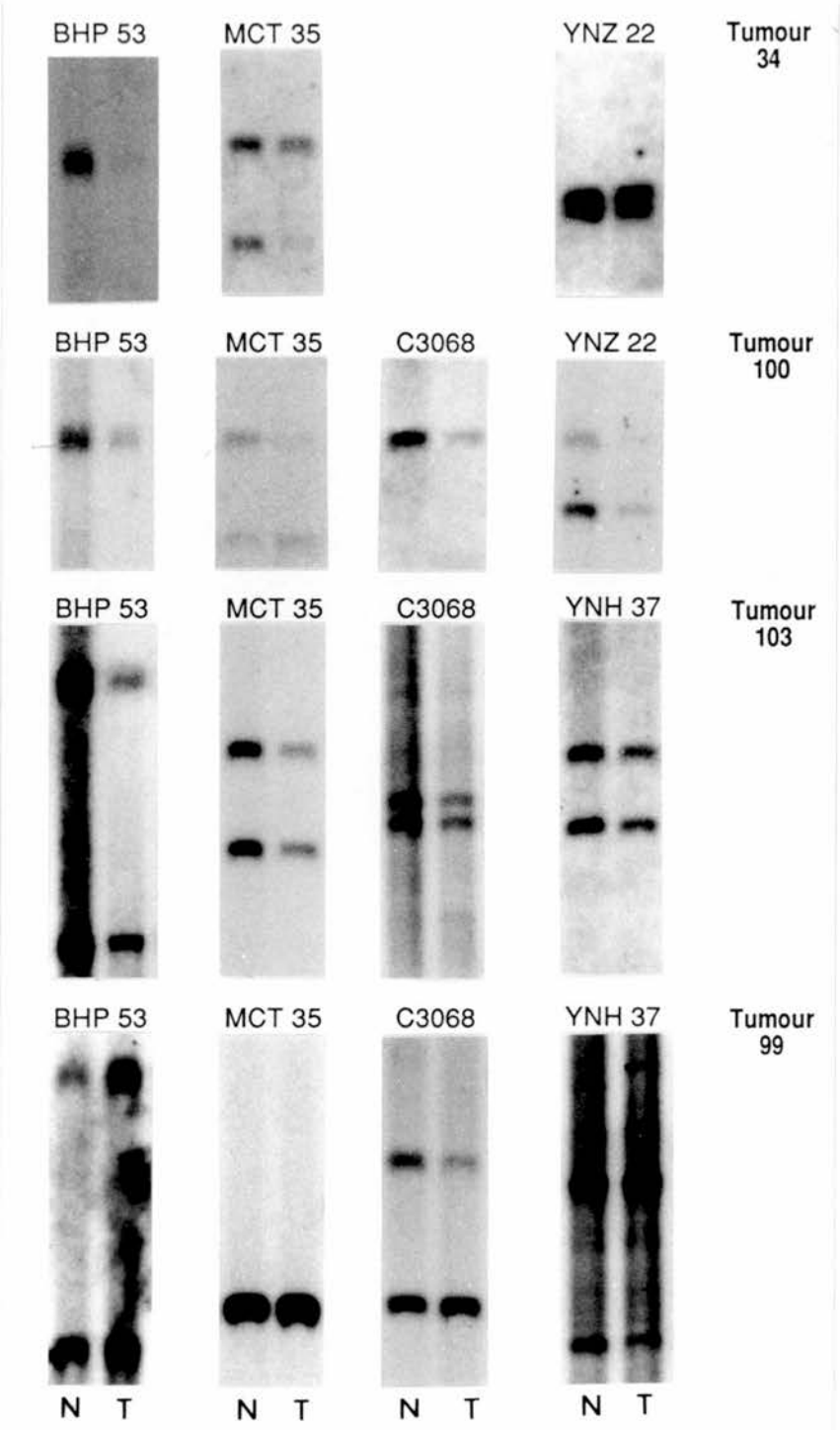
as 75% in comparison to a LOH frequency of 48% at 17p13.1 loci. The data from this study, when analysed visually and combined with the data from Mackay *et al.* (1988a), also indicated a difference in the frequency of LOH between the two loci (Coles *et al.*, 1990). However due to the limited number of tumours available for quantitative analysis at the YNZ22/D17S5 and YNH37/D17S28 loci these data can no longer show this difference in LOH frequency. Similar frequencies of LOH are observed at the BHp53/TP53 (44%), MCT35/D17S31 (56%), c3068/- (53%) and YNH37/D17S28 (50%) loci (Table 3.3).

3.8 Determination of a shortest region of overlap.

The determination of a SRO involves the comparison of LOH status at adjacent loci. Although using the 40% level of significance to determine LOH frequency is adequate when analysing single loci, simply using this when analysing markers on the same chromosome arm would be an unsatisfactory way of producing mapping data. For example, analysis of LOH at the 40% level in tumour 14 would classify the tumour as showing LOH at the MCT35/D17S31 loci but not at the BHp53/TP53 and c3068/ loci (Table 3.2). The difference in relative allele intensity reduction in this tumour is only 7% between the BHp53/TP53 and MCT35/D17S31 loci and 15% between the MCT35/D17S31 and c3068/- loci and could be due to experimental variation alone. For this reason, this tumour sample would be unsuitable for determining a SRO. In order to avoid this problem, only those tumour samples showing a difference in allele intensity ratios between adjacent loci above a certain level would be selected for the purpose of mapping.

If the data are analysed by selecting only those tumours in which adjacent loci show a difference in allele intensity ratios greater than a value of 40%, then only tumours 34 and 100 would be suitable for determining an SRO. Tumours 34 and 100 show a difference in relative allele intensity ratios between the MCT35/D17S31 and YNZ22/D17S5 markers of 55% and 78% respectively (Figure 3.4, Table 3.2) and indicate a LOH event occurring somewhere proximal to the YNZ22/D17S5 marker in both tumour samples. This suggests that the inactivation of a tumour suppressor gene proximal to the YNZ22/D17S5 locus contributed to the development of both tumours. In order to determine an SRO in these tumours other chromosome 17p loci were

Figure 3.4 Southern blot analysis of four pairs of constitutional (N) and tumour (T) DNA using four chromosome 17p probes. Tumours 34 and 100 show LOH with MCT35 but not with YNZ22. Tumour 103 shows no LOH at all four loci. Tumour 99 shows LOH at all informative loci.



investigated for LOH. By using probes mapping between the YNZ22/D17S5 and MCT35/D17S31 loci and proximal to the MCT35/D17S31 locus, further data on the extent of the region of LOH could be produced. Unfortunately the analysis of tumour 34 with the other chromosome 17p markers was not possible due to the limited amount of tumour DNA available. Tumour 100 was analysed at the c3068/ and BHp53/TP53 loci but was found to be uninformative for both of these markers (Table 3.2, Figure 3.4). Additional chromosome 17p probes would need to be obtained before further mapping with tumour 100 could continue.

Following the discovery the high frequency of LOH at the YNZ22/D17S5 locus in breast cancer, a number of groups have attempted to define a SRO on chromosome 17p (Coles *et al.*, 1990; Devilee *et al.*, 1990; Sato *et al.*, 1991a; Andersen *et al.*, 1992). The earlier study by Devilee *et al.* (1990) determined a single SRO defined by the loci EW504/D17S67, which maps to 17p12 and YNZ22/D17S5. The three later studies, using additional probes, determined two SROs on chromosome 17p, suggesting the presence of two genes involved in breast tumorigenesis. The study by Sato *et al.* (1991a) implicated one of the regions between the marker LB17.3, which maps to 17p13.1 - 17p13.3, and the telomere of the chromosome and the other between the marker BHp53/TP53 and the centromere. The study by Andersen *et al.* (1992) further localised the telomeric SRO between the 144D6/D17S34 and YNZ22/D17S5 loci, a region 7kb in length (Y. Nakamura, personal communication). The second SRO was localised between the YNZ22/D17S5 loci and the centromere. Evidence for two SROs on chromosome 17p was also generated when the data from the present study and that of Mackay *et al.* (1988a) were analysed visually (Coles *et al.*, 1990). However due to the limited number of tumours available for quantitative analysis at the YNZ22/D17S5 locus the data are inadequate to illuminate the issue of two SROs on chromosome 17p.

3.9 Discussion.

As the location of the p53 gene falls within the more proximal SRO region determined by both Sato *et al.* (1991a) and Andersen *et al.* (1992) it is thought that LOH in this area may indicate

the inactivation of one copy of the p53 gene. To date the only gene sequence known to map to the 17p13.3 region is that of the profilin gene which codes for an actin monomer binding protein (Kwiatkowski *et al.*, 1990). Unfortunately it is located proximal to the YNZ22 locus and is therefore outside the SRO determined by Andersen *et al.* (1992). However it is possible that there is more than one tumour suppressor gene located at 17p13.3 and, until the gene has been screened for alterations in tumours, a role for the profilin gene in breast tumorigenesis cannot be excluded.

Detailed studies of patterns of LOH in colon cancer have identified only a single SRO, which contains the p53 gene (Baker *et al.*, 1989). LOH studies of hepatocellular carcinomas (Fujimori *et al.*, 1991), renal cell carcinomas (Morita *et al.*, 1991), medulloblastomas (Cogen *et al.*, 1992), ovarian carcinomas (Sato *et al.*, 1990) and neuroectodermal tumours (Biegel *et al.*, 1992) have revealed a pattern of loss similar to that observed in breast cancer ie loss of telomeric sequences on chromosome 17p without apparent loss of the p53 gene. This suggests that the putative tumour suppressor gene at 17p13.3 may play a part in the development of a number of malignancies and that identification of this gene may not have to rely on mapping data produced from breast cancer alone.

Ideally LOH mapping in breast cancer samples requires tumour DNA derived from samples with little contamination by normal tissue and in which tumour cells contain the same genetic alterations. Of the 40 tumours analysed at one or more loci only three samples, tumours 18, 21 and 101, showed a complete loss of an allele (Table 3.2). This suggests that the tumour samples used in the LOH assay were frequently contaminated by normal tissue or contained more than one type of clone. Consequently few mapping data were able to be produced from this series of experiments. In future, histological analysis of all tumour material before use in mapping experiments would identify those samples containing a high level of normal cell contamination and would enable the identification of samples showing tumour heterogeneity (Lundberg *et al.*, 1987; Devilee *et al.*, 1990; Larsson *et al.*, 1990).

The mapping data generated identify two tumour samples both showing LOH at a site near to the p53 gene but not at more distal loci. LOH is observed at loci near to the p53 gene in 17/35 (49%) of samples. These two observations suggest a role for the p53 gene in breast tumorigenesis

and further experiments investigating the status of the p53 gene in this bank of tumour DNA samples were undertaken.

Chapter 4

Detection of mutations in the p53 gene using the amplification and mismatch detection (HOT) technique

4.1 Mutation detection techniques.

The determination of a single SRO between 17p12 and 17p13.3 and the close correlation of LOH on chromosome 17 with the mutation of the remaining p53 allele in colon and other cancers, strongly suggested that the tumour suppressor gene inactivated during the development of those malignancies was the p53 gene (Baker *et al.*, 1989; Nigro *et al.*, 1989; Baker *et al.*, 1990a). Due to the high frequency of LOH near the p53 locus observed in the previous set of experiments and the detection of p53 mutations in breast cancer cell lines (Nigro *et al.*, 1989; Bartek *et al.*, 1990a) the breast tumour samples were screened for the presence of p53 mutations. Early mutation studies on the p53 gene relied on the isolation of mRNA and the synthesis and sequencing of p53 cDNA from tumour samples, a process both labour intensive and time consuming (Baker *et al.*, 1989; Nigro *et al.*, 1989). More recent studies, especially those examining a large number of tumour samples, have relied on non-sequencing techniques to identify p53 mutations before sequencing is attempted.

Those techniques most frequently used are SSCP (single strand conformation polymorphism) (Orita *et al.*, 1989; Spinardi *et al.*, 1991), DGGE (denaturing gradient gel electrophoresis) (Sheffield *et al.*, 1989) and modifications of it, CDGE (constant denaturing gel electrophoresis) (Borresen *et al.*, 1991) and HOT (also known as the amplification and mismatch detection technique or chemical cleavage technique; named after the modifying chemicals used, hydroxylamine hydroxide and osmium tetroxide). SSCP relies on the differing conformations adopted by single stranded DNA molecules of different sequence which result in an alteration in electrophoretic mobility. Regions of interest are simply amplified, denatured and run out on a non-denaturing polyacrylamide gel with a normal control. DGGE/CDGE is based on strand separation of DNA fragments in discrete sequence-dependent melting domains. Strand separation results in a decrease in the electrophoretic mobility of the DNA fragment as it moves through a polyacrylamide gel containing an increasing (DGGE) or constant (CDGE) gradient of denaturant. Both SSCP and DGGE/CDGE techniques are sensitive enough to separate DNA fragments differing in only a single base.

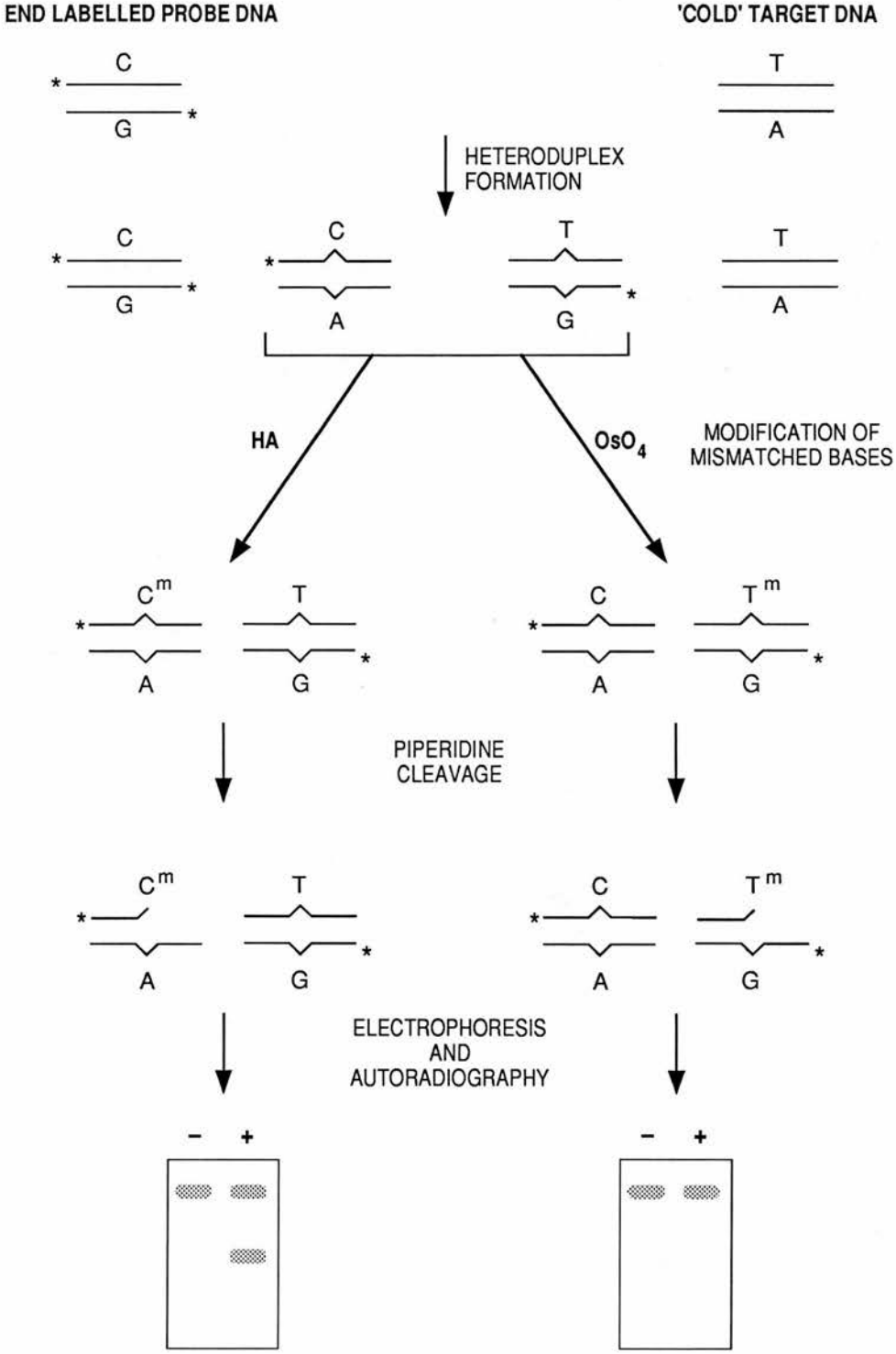
4.2 The HOT technique.

The HOT technique was developed by Cotton *et al.*, (1988) and is based on the principles of Maxam and Gilbert sequencing (Maxam and Gilbert 1977). The basis of the technique is as follows. Wild type and mutant DNA sequences are mixed together, denatured into single strands and allowed to renature to form a wild type/mutant heteroduplex. Regions of mismatch are produced within the heteroduplex molecule wherever the two sequences differ. The mismatched sequences are modified, either by hydroxylamine hydroxide (HA) or osmium tetroxide (OsO_4), followed by chemical cleavage of the sugar-phosphate backbone at these modified sites by piperidine. HA modifies across the 5,6 double bond of cytosine bases, OsO_4 modifies across the 5,6 double bond of thymine bases. Cleaved products can be separated from full length fragments on a denaturing polyacrylamide gel and, when using radioactively labelled DNA fragments, identified by autoradiography (Figure 4.1).

The HOT technique as described in section 2.10 is carried out by mixing radioactively end-labelled DNA fragments (known as the probe DNA) with unlabelled fragments from a different source (known as the target DNA). Since detection is dependent on heteroduplex formation, the probe DNA, which can be either wild type or mutant, is mixed with approximately ten times the amount of target DNA prior to denaturing. This ensures that nearly all of the probe DNA forms a heteroduplex rather than a homoduplex. For convenience, when scanning a large number of DNA samples for mutations, wild type probe DNA is used to screen the sample target DNAs. Using both HA and OsO_4 it is theoretically possible to detect all types of mutations using wild type probe DNA with target DNA (Table 4.1).

The HOT technique has two main advantages over the other non-sequencing techniques. Firstly, DNA fragments greater than 1kb can be analysed for mutation (Cotton 1992). Both SSCP and DGGE/CDGE techniques have a size limit of about 400bp (Grompe *et al.*, 1989; Orita *et al.*, 1990). Secondly, since chemical cleavage occurs at the site of mismatch, the size of the cleaved fragment indicates how many bases into the fragment the mutation is located. Once a mismatch has been detected the mutation can be located in one of two places so subsequent sequencing in order to define the specific change is minimal. In addition, once the specific mutation has been identified it can be confirmed by comparing the expected and observed fragment sizes following HA or OsO_4

Figure 4.1



Detection of a GC > TA substitution using the HOT technique.
C^m and T^m represent bases modified by Hydroxylamine (HA) and Osmium tetroxide (OsO_4) respectively.
Negative controls(-) are produced when the target and probe DNAs are identical.

modification and piperidine cleavage. This is particularly useful when detecting mutations in material containing both mutant and wild type sequences.

Table 4.1 Base change detection by the HOT technique using wild type probe DNA and mutant target DNA.

Base change	Labelled wild type probe DNA	Unlabelled mutant target DNA	Mismatched bases	Mismatch detection using HA	Mismatch detection using OsO_4
GC>AT	*G*C	AT	*GT *CA	Negative Positive	Negative Negative
GC>TA	*G*C	TA	*GA *CT	Negative Positive	Negative Negative
GC>CG	*G*C	CG	*GG *CC	Negative Positive	Negative Negative
AT>GC	*A*T	GC	*AC *TG	Negative Negative	Negative Positive
AT>CG	*A*T	CG	*AG *TC	Negative Negative	Negative Positive
AT>TA	*A*T	TA	*AA *TT	Negative Negative	Negative Positive

*N - Base on end-labelled fragment

4.3 Detection of mismatches in p53 DNA fragments amplified from primary breast tumours.

Previous work in other malignancies identified the majority of p53 mutations in exons 5-8 of the p53 gene (Nigro *et al.*, 1989; Hollstein *et al.*, 1991a; Caron de Fromentel *et al.*, 1992). Breast tumour samples were therefore screened for p53 mutations in this region of the gene. Oligonucleotide primers were used to amplify exons 5 and 6 in a 408bp fragment (fragment III) and exons 7, 8 and 9 in a 792bp fragment (fragment IV) from 78 primary breast tumour samples. End-labelled wild type probe DNA was mixed with an excess of unlabelled tumour target DNA from each tumour sample, denatured by heat and cooled to form the probe/target heteroduplex. Following HA and OsO_4 modification and piperidine cleavage the samples were loaded, with a positive control,

onto a denaturing polyacrylamide gel. The resulting autoradiographs were analysed for the presence of any mismatches (Figures 4.2, 4.3, 4.4, 4.5).

Mismatches were detected in 19 tumours in fragment III and 23 tumours in fragment IV. Analysis of the cleaved fragments bands indicated that 18 of the 80 tumours contained mismatches which could be attributed to two known polymorphisms. One of the polymorphisms was detected in fragment IV and is known to be located in intron 7. It is are comprised of two substitutions, GC>AT and AT>CG, which occur 72 and 92 nucleotides into intron 7 respectively (Prosser and Condie 1991). Using wild type probe DNA and tumour target DNA the polymorphism is identified by a 197bp fragment after HA modification and a 217bp fragment after OsO_4 modification (Figure 4.6). Due to the high frequency and previous characterisation of the polymorphism by the HOT technique the corresponding lymphocyte DNA was not sequenced to confirm the presence of the polymorphism. The polymorphism was found in 6/60 (10%) breast cancer patients in the study by Prosser and Condie (1991) and 16/73 (22%) breast cancer patients in this study.

The second polymorphism was detected in fragment III and is located in exon 6. This polymorphism was detected in 2 of 78 tumours and is known to correspond to a AT>GC substitution at the third nucleotide of codon 213 (Carbone *et al.*, 1991; Mazars *et al.*, 1992a). It is a silent alteration and results in no amino acid change in the p53 protein. Using wild type probe DNA and tumour target DNA the polymorphism is identified by a 49bp cleaved fragment after OsO_4 modification (Figure 4.6). The presence of the sequence alteration in somatic cells was confirmed by sequencing fragment III amplified from the corresponding patient lymphocyte DNA (Figure 4.7). The polymorphism was detected in 2 of 73 (3%) patients, a frequency similar to that found in other studies: 3% (6/189) (Carbone *et al.*, 1991); 3% (2/70) (Mazars *et al.*, 1992a); 3% (1/34) (Hollstein *et al.*, 1991b); (4%) 1/26 (Osborne *et al.*, 1991); 3% (1/33) (Bodner *et al.*, 1992); and 5% (1/22) (von Deimling *et al.*, 1992).

4.4 Characterisation of the p53 mutations.

In total 24 mismatches were identified, excluding those caused by the polymorphisms in exon 6 and intron 7. Seventeen of these mismatches were identified in fragment III, 7 were detected

Figure 4.2 Detection of mismatches in exons 5 and 6 of the p53 gene in primary breast tumours: HA modification and piperidine cleavage of heteroduplexes between wild type probe DNA and tumour target DNA. Arrows indicate the positions of cleaved fragments. Tumour 34 (T34) was used as a positive control.

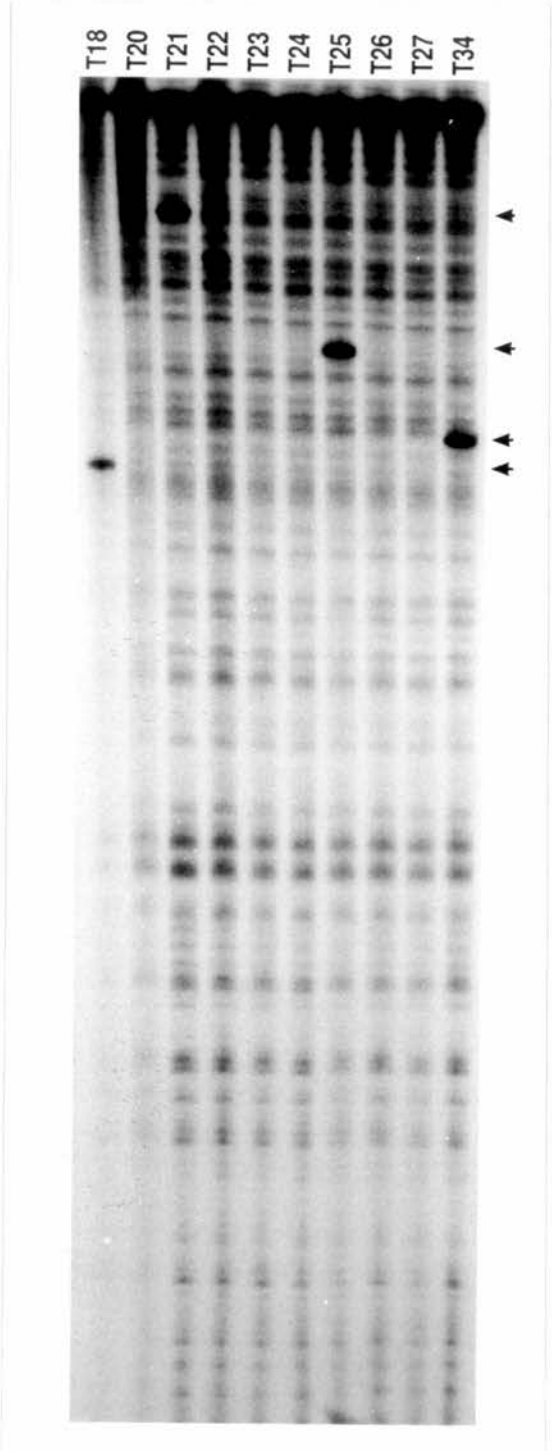


Figure 4.3 Detection of mismatches in exons 5 and 6 of the p53 gene in primary breast tumours: OsO_4 modification and piperidine cleavage of heteroduplexes between wild type probe DNA and tumour target DNA. Arrows indicate the positions of cleaved fragments. Tumour 34 (T34) was used as a positive control.

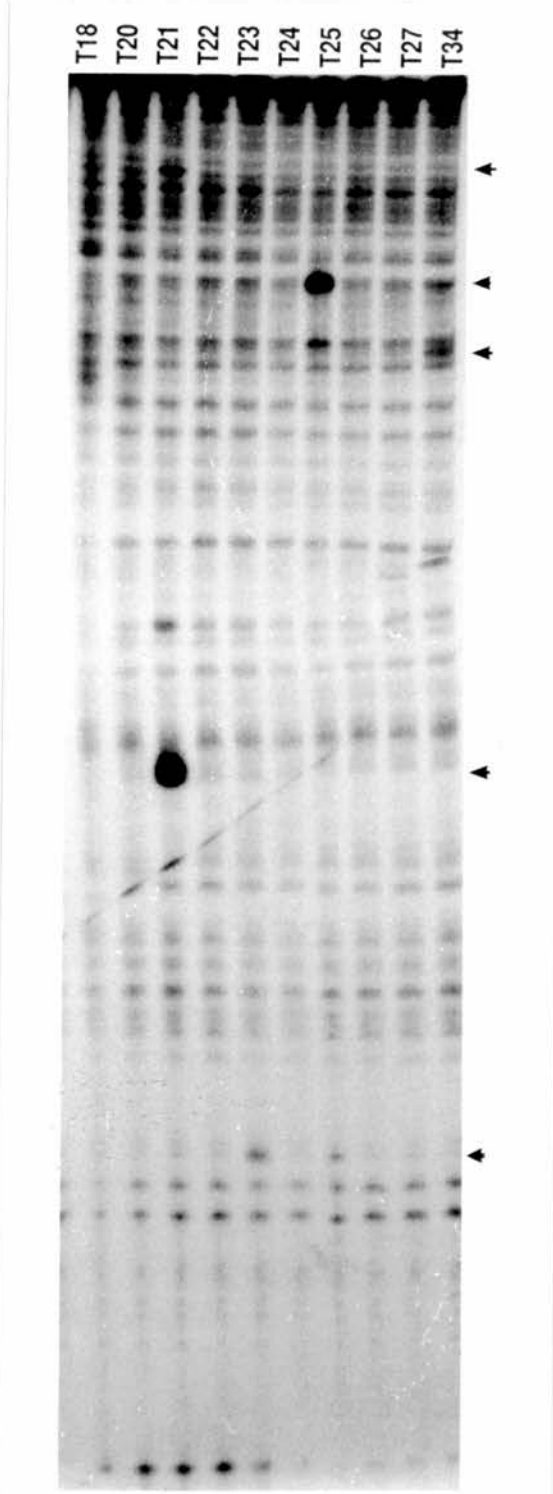


Figure 4.4 Detection of mismatches in exons 7, 8 and 9 of the p53 gene in primary breast tumours: HA modification and piperidine cleavage of heteroduplexes between wild type probe DNA and tumour target DNA. Arrows indicate the positions of cleaved fragments. Tumour 9 (9T) was used as a positive control.

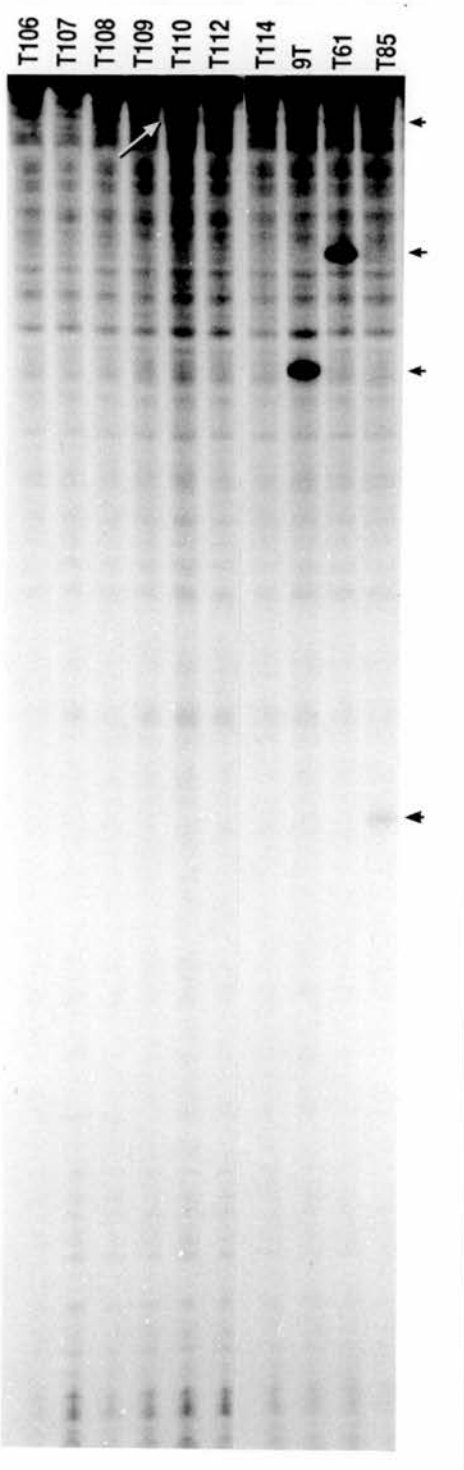


Figure 4.5 Detection of mismatches in exons 7, 8 and 9 of the p53 gene in primary breast tumours: OsO_4 modification and piperidine cleavage of heteroduplexes between wild type probe DNA and tumour target DNA. Arrows indicate the positions of cleaved fragments. Tumour 9 (9T) was used as a positive control.

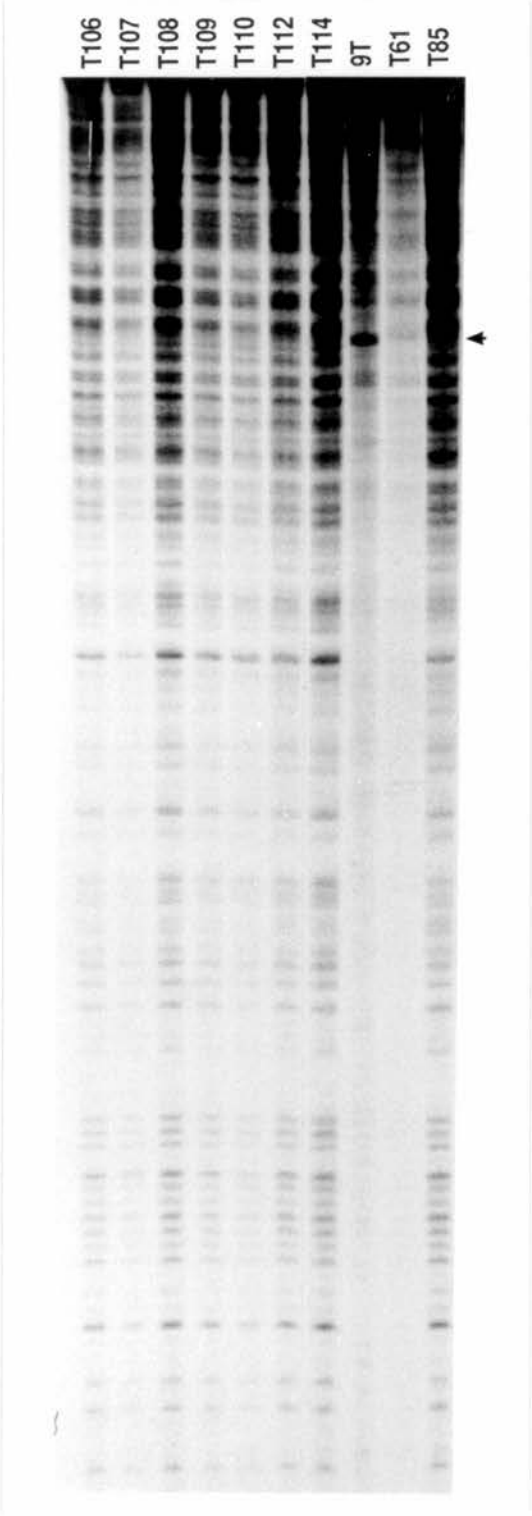


Figure 4.6 Detection of the intron 7 and exon 6 polymorphisms of the p53 gene by the HOT technique. The intron 7 polymorphism is present in tumour 99. The exon 6 polymorphism is present in tumour 74.

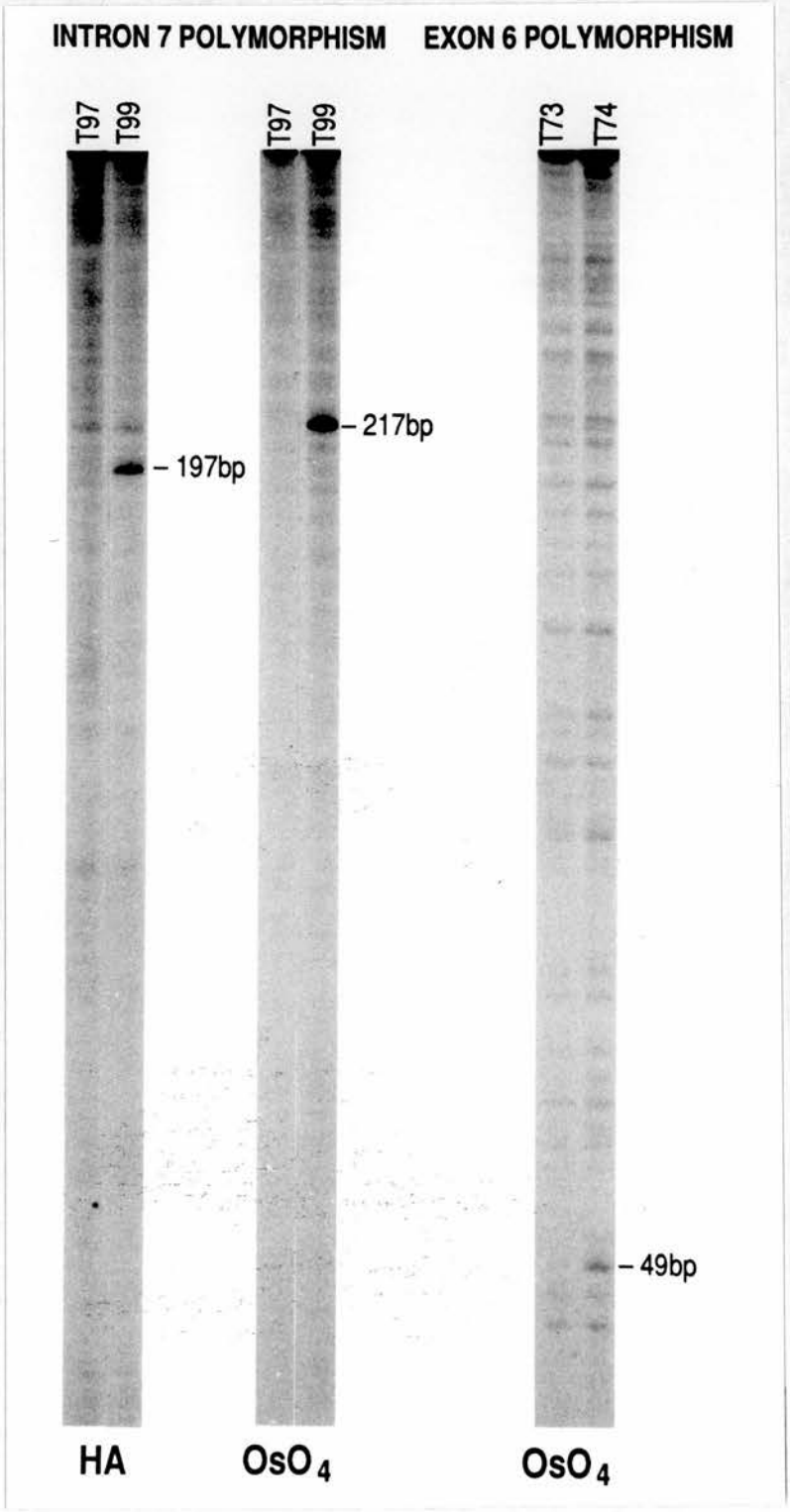
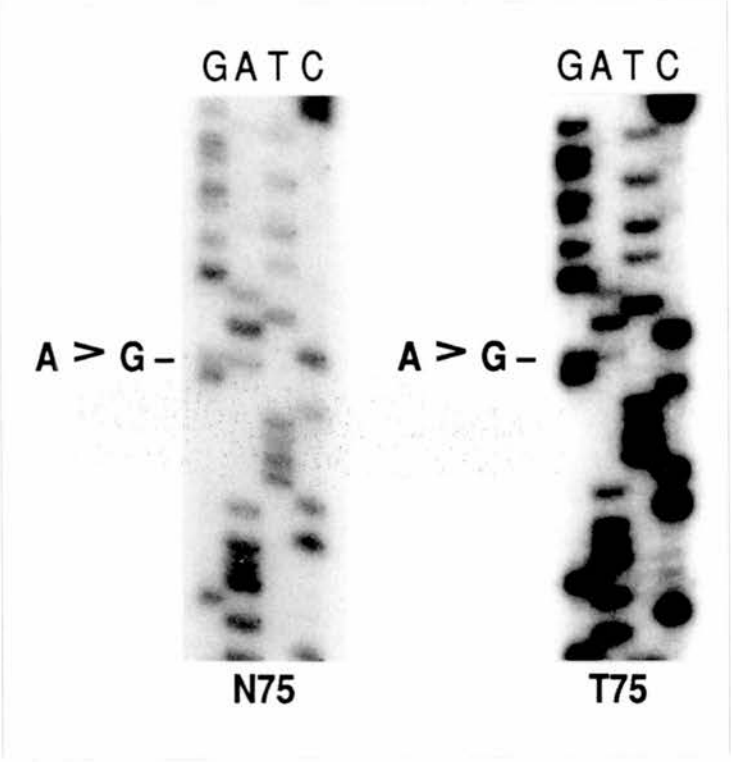


Figure 4.7 Sequence of the codon 213 polymorphism determined in both tumour 75 (T75) and paired normal DNA (N75).



in fragment IV. A greater frequency of p53 mutations has been detected in exons 7 and 8 than in exons 5 and 6 in a number of breast cancer studies (section 5.3.5). Since fewer mutations were detected in fragment IV than in fragment III in this tumour series, fragment IV samples were reanalysed by the HOT technique using tumour probe DNA and wild type target DNA. An additional mismatch was detected in a single tumour, tumour 98, which had not been identified when using wild type probe DNA and tumour target DNA (Figure 4.8).

In order to estimate the size of the cleaved fragments, and so help pinpoint the area where the mutations reside, the detection of the mismatches by the HOT technique was repeated. The samples were run out on a denaturing polyacrylamide sequencing gel with either p53 fragment III or IV sequencing reaction products as a size marker (Figures 4.9, 4.10, 4.11). Once the fragment sizes were determined, sequencing was carried out from either end and the appropriate area examined for mutation. Mismatch analysis and sequencing were performed on independently amplified PCR fragments to ensure that the mutations identified had not arisen from infidelity of the taq polymerase enzyme. A change in sequence was identified in 24 of the 25 tumour samples identified by the HOT technique (Figure 4.12, Tables 4.2, 4.3, 4.4).

In one case, tumour 85, a mismatch was detected in fragment IV resulting in a cleaved fragment length of approximately 80bp after HA modification (Figures 4.4, 4.13). Sequencing of the fragment detected no mutation 80bp from either end of the fragment (Figure 4.14). Reanalysis of two further independently amplified fragments confirmed the presence of the 80bp fragment. In an effort to identify the possible type of base change present in the p53 gene of this tumour, mutation detection by the HOT technique was performed using tumour probe DNA and wild type target DNA. No cleaved bands were observed on the resulting autoradiograph (Figure 4.13). This pattern is consistent with a CG>TA substitution. The 80bp cleaved fragment is generated from a CA mismatch when the wild type fragment is end labelled. The absence of any cleaved product when the mutant fragment is labelled can be explained by the insufficient modification of a GT mismatch (section 4.5). However, repeated sequencing of independently amplified samples detected no mutation at any site on the fragment.

Figure 4.8 Detection of mismatches in exons 7, 8 and 9 in the p53 gene in primary breast tumours: HA modification and piperidine cleavage of heteroduplexes between tumour probe DNA and wild type target DNA. The 585bp cleaved fragment represents the intron 7 polymorphism in tumours 98 and 99. The 721bp fragment represents the AT>GC substitution present in tumour 98.

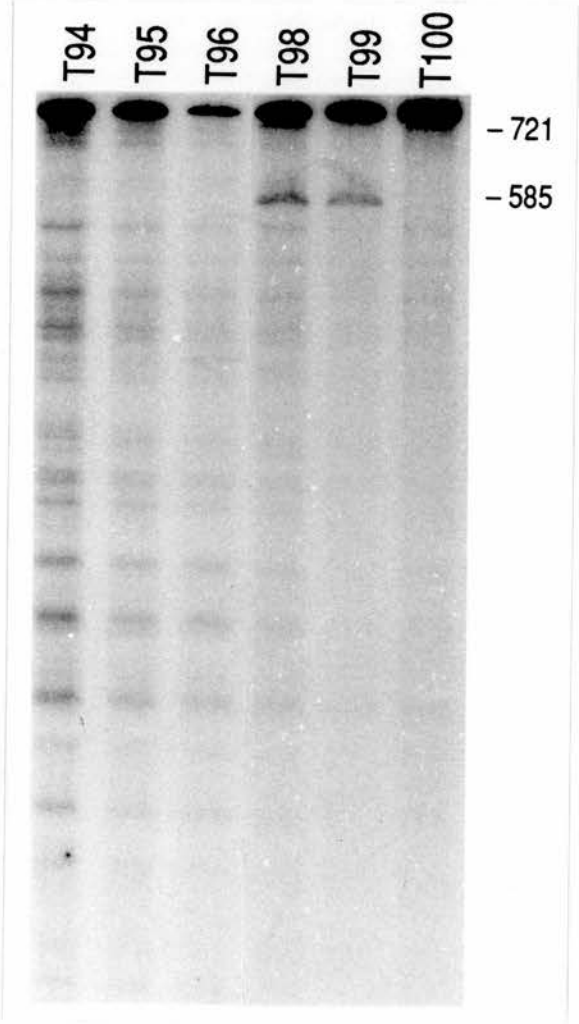


Figure 4.9 Detection of mismatches in exons 5 and 6 of the p53 gene in primary breast tumours: size determination of HA modified and piperidine cleaved fragments. p53 fragment III sequencing reaction products, used as size markers, are indicated by G, A, T, C.

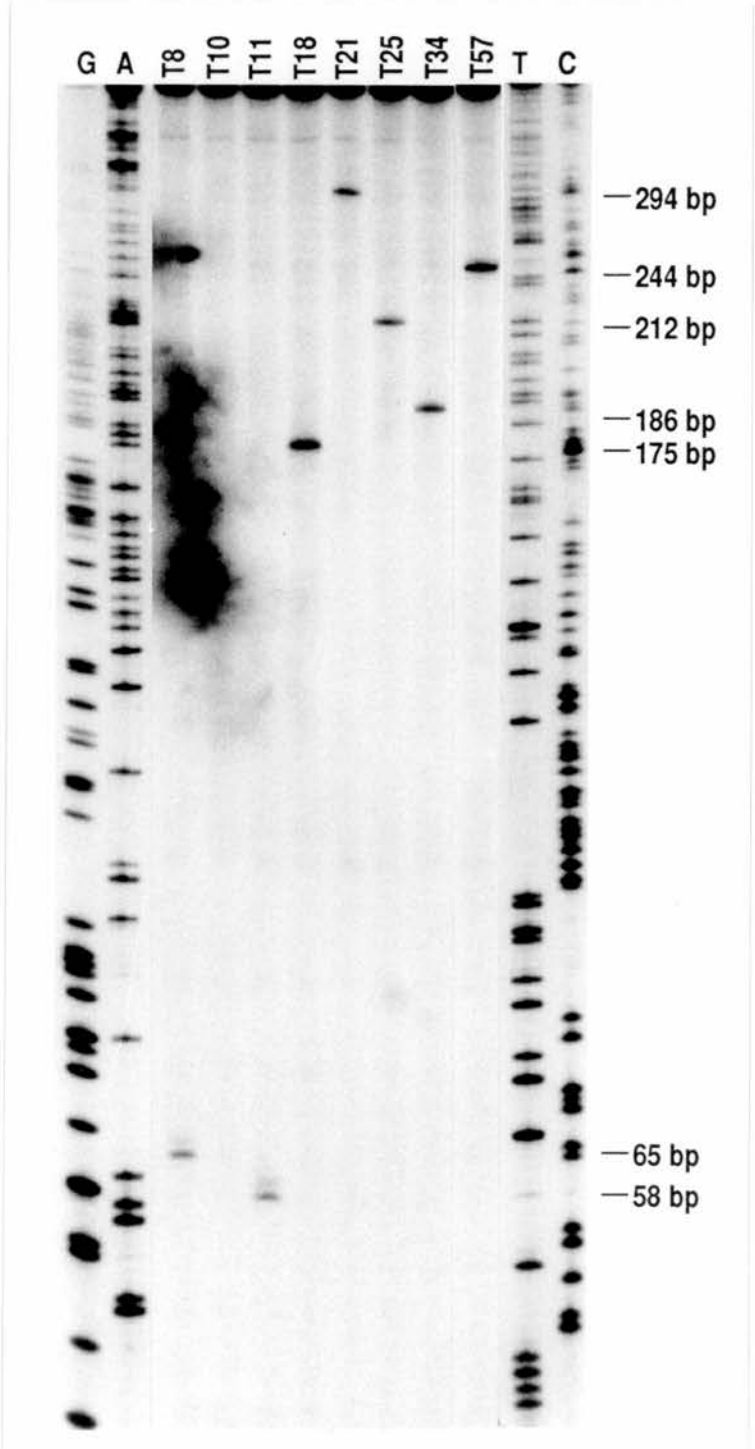


Figure 4.10 Detection of mismatches in exons 5 and 6 of the p53 gene in primary breast tumours: size determination of OsO_4 modified and piperidine cleaved fragments. p53 fragment III sequencing reaction products, used as size markers, are indicated by G, A, T, C.

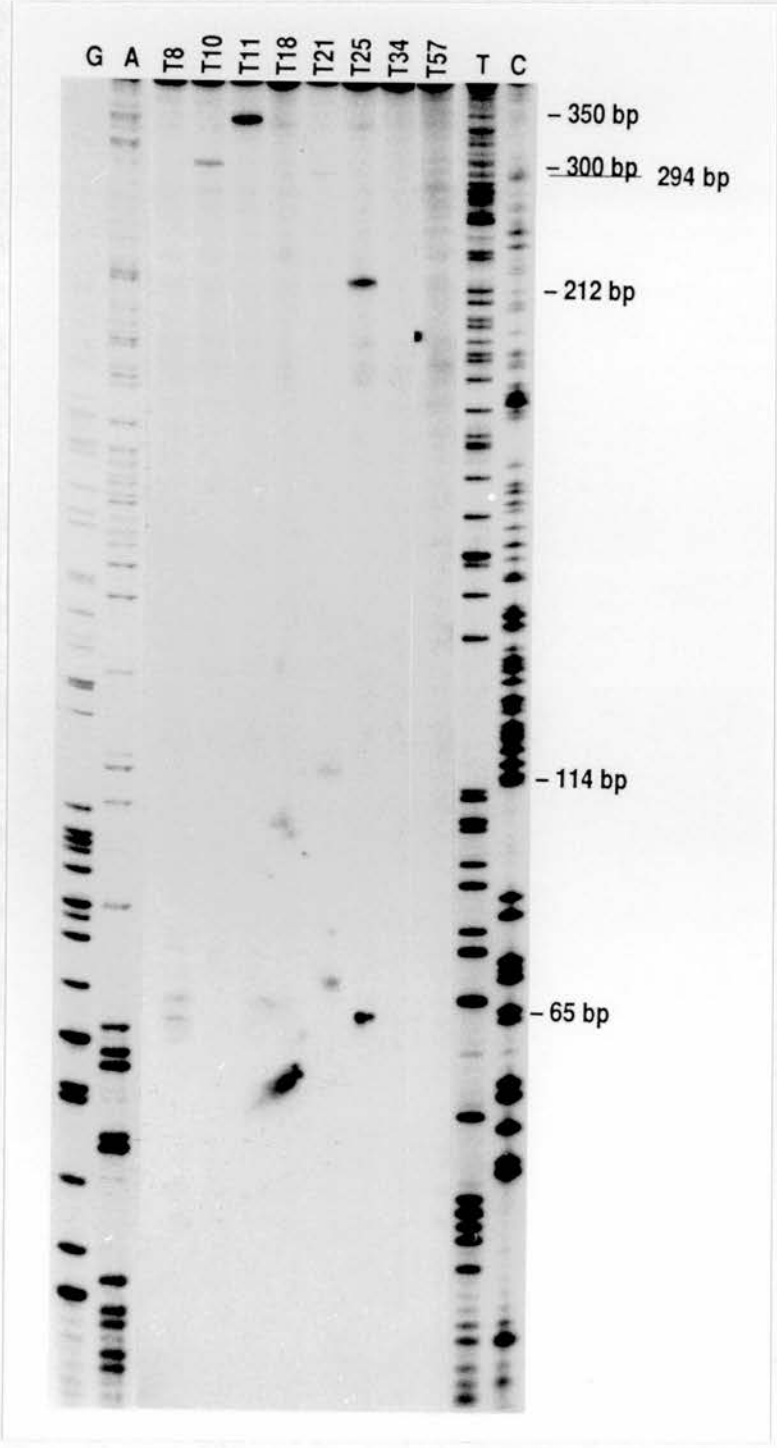


Figure 4.11 Detection of mismatches in exons 7, 8 and 9 of the p53 gene in primary breast tumours: size determination of HA and OsO_4 modified and piperidine cleaved fragments. $T8^H$, $T12^H$, etc indicate heteroduplexes modified by HA, $T8^O$, $T92^O$, etc represent heteroduplexes modified by OsO_4. p53 fragment IV sequencing reaction products, used as size markers, are indicated by G, A, T, C.

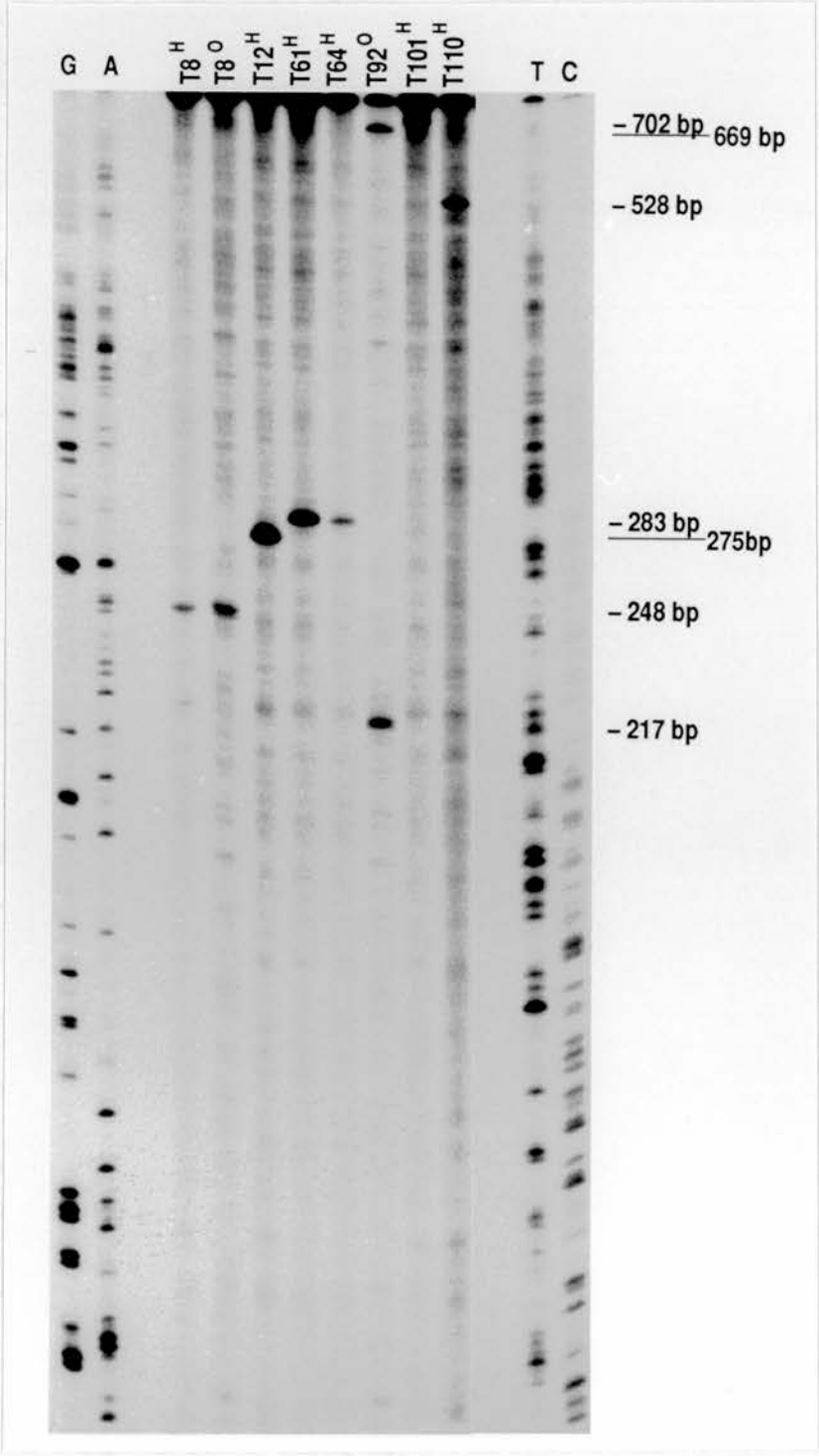


Table 4.2 Chemical modification of mismatched bases detected in exons 5 and 6 of the p53 gene in primary breast tumours using wild type probe DNA.

Tumour Number	Nucleotide change	Mismatched bases	Expected Bands	Observed Bands	Possible Explanation of Unexpected Bands
8	GAC > GTC	AA and TT	HA: - Os: 65	HA: 65 Os: 65	C adjacent to TT mismatch
10/72/ 113	CTT > CGT	CT and GA	HA: - Os: 300	HA: - Os: 300	As expected
11	GAC > GΔC	A Δ and Δ T	HA: - Os: 350	HA: 58 Os: 350	C adjacent to AΔ mismatch
18	CCA > CTA	CA and GT	HA: 175 Os: -	HA: 175 Os: -	As expected
21	TCA > TTA	CA and GT	HA: 294 Os: -	HA: 294 Os: 294/114	T adjacent to CA mismatch
25	CGA > CTA	GA and CT	HA: 212 Os: -	HA: 212 Os: 212	T adjacent to CT mismatch
34	GCT > GAT	CT and GA	HA: 186 Os: -	HA: 186 Os: 186	T adjacent to CT mismatch
57/114	CGC > CAC	GT and CA	HA: 244 Os: -	HA: 244 Os:-	As expected
64/95	TCG > TTG	CA and GT	HA: 357 Os: -	HA: 357 Os:-	As expected
67	CTA > CAA	TT and AA	HA: - Os: 127	HA: - Os: 127/281	T adjacent to AA mismatch
71	TGC > TAC	GT and CA	HA: 336 Os: -	HA: 336 Os: 336	No T adjacent to CA mismatch
Exon 6 Poly	GAC > GGC	GT and CA	HA: - Os: 48	HA: - Os: 48	As expected

Poly - Polymorphism
Δ - single base deletion

Table 4.3 Chemical modification of mismatch bases detected in exons 7, 8 and 9 of the p53 gene in primary breast tumours using wild type probe DNA.

Tumour Number	Nucleotide change	Mismatched bases	Expected Bands	Observed Bands	Possible Explanation of Unexpected Bands
8	AGA > AAA	GT and CA	HA: 248 Os: -	HA: 248 Os: 248	T adjacent to CA mismatch
12	TGC > TCC	GG and CC	HA: 275 Os: -	HA: 275 Os: -	As expected
61	CGT > CAT	GT and CA	HA: 283 Os: -	HA: 283 Os: -	As expected
84	CGT > CTT	GA and CT	HA: 283 Os: -	HA: 283 Os: -	As expected
85	Not Known	Not Known	HA: NK Os: NK	HA: 80 Os: -	-
88	TGT > TTT	GA and CT	HA: 725 Os: -	HA: 725 Os: 55	T adjacent to GA mismatch
92	TCA > Δ Δ Δ	TΔ and CΔ and AΔ	HA: 111 Os: 111/669	HA: 111 Os: 111/669	As expected
98	TAA > TGA	CA and GT	HA: - Os: 721	HA: - Os: -	GT mismatch unmodified
101	GGC > GAC	GT and CA	HA: 702 Os: -	HA: 702 Os: -	As expected
110	CCG > CTG	CA and GT	HA: 523 Os: -	HA: 523 Os: -	As expected
Intron 7 Poly	GCC > GTC	CA and GT	HA: 197 Os: -	HA: 197 Os: -	As expected
Intron 7 Poly	TTC > TGC	GA and CT	HA: - Os: 217	HA: - Os: 217	As expected

Poly - Polymorphism
NK - Not Known
Δ - single base deletion

Table 4.4 Chemical modification of mismatch bases detected in exons 7, 8 and 9 of the p53 gene in primary breast tumours using mutant probe DNA.

Tumour Number	Nucleotide change	Mismatched bases	Expected Bands	Observed Bands	Possible Explanation of Unexpected Bands
8	AGA > AAA	GT and CA	HA: - Os: 248	HA: ND Os: ND	-
12	TGC > TCC	GC and CG	HA: 515 Os: -	HA: 515 Os: 515	T adjacent to CC mismatch
61	CGT > CAT	GT and CA	HA: - Os: 283	HA: - Os: 283/497	C adjacent to AC mismatch
84	CGT > CTT	GA and CT	HA: - Os: 497	HA: ND Os: ND	-
85	Not Known	Not Known	HA: NK Os: NK	HA: 80 Os: -	-
88	TGT > TTT	GA and CT	HA: - Os: 55	HA: - Os: 55	As expected
92	TCA > Δ Δ Δ	TΔ and CΔ and AΔ	HA: - Os: -	HA: - Os: 669/111	Two Ts adjacent to TCA Δ Δ Δ mismatch
98	TAA > TGA	CA and GT	HA: 721 Os: -	HA: 721 Os: -	As expected
101	GGC > GAC	GT and CA	HA: - Os: 702	HA: - Os: -	GT mismatch
110	CCG > CTG	CA and GT	HA: - Os: 257	HA: - Os: -	GT mismatch
Intron 7 Poly	GCC > GTC	CA and GT	HA:- Os:197	HA:- Os:-	GT mismatch
Intron 7 Poly	TTC > TGC	GA and CT	HA:585 Os:-	HA:585 Os:-	As expected

Poly - Polymorphism
ND - Not Done
NK - Not Known
Δ - single base deletion

Figure 4.12 Sequence characterisation of p53 mutations detected in eight breast tumours by the HOT technique.

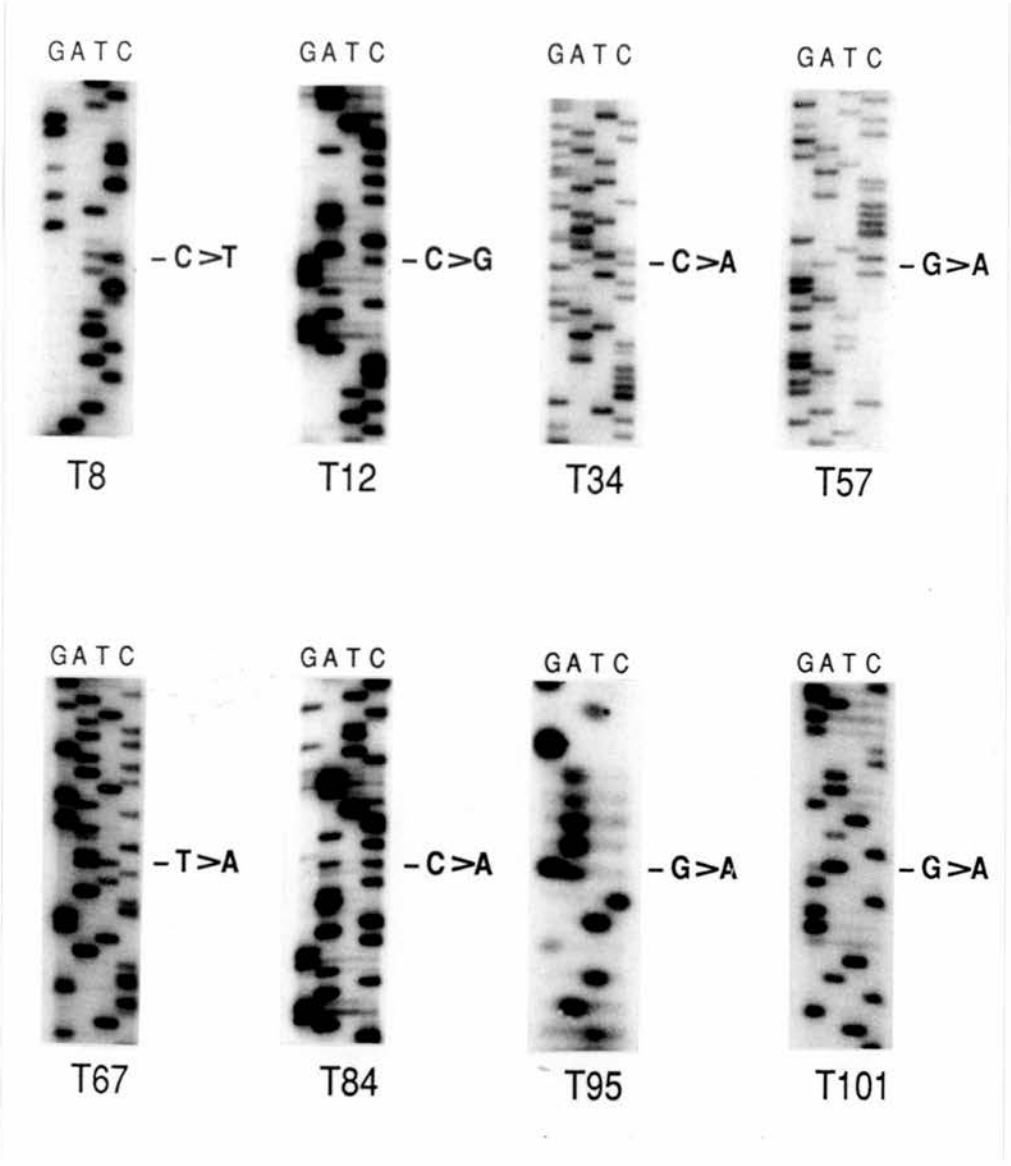


Figure 4.13 Mismatch detection in tumour 85 using the HOT technique. An 80bp fragment is detected using wild type probe and tumour target DNA. No cleaved fragments are detected using tumour probe and wild type target DNA.

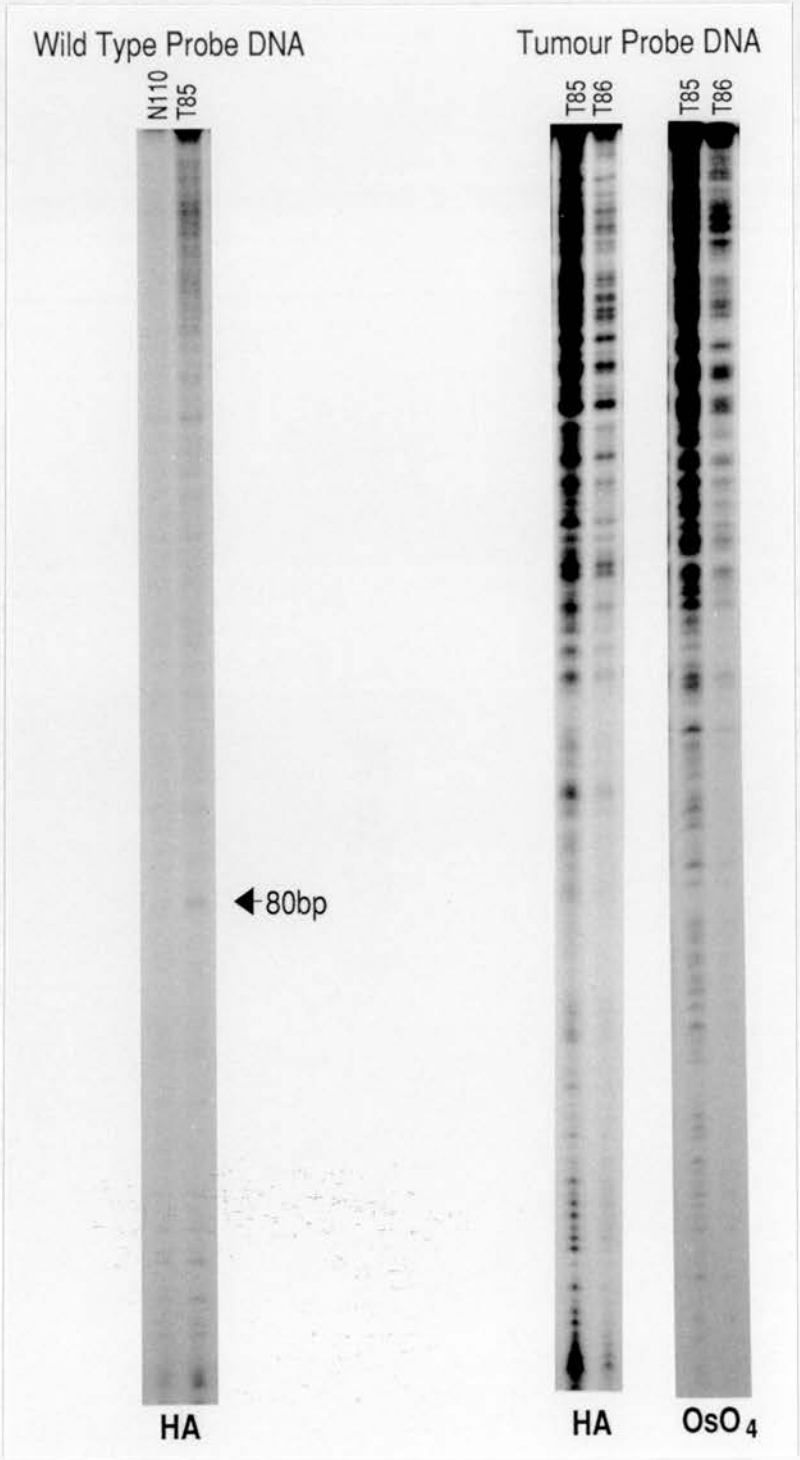
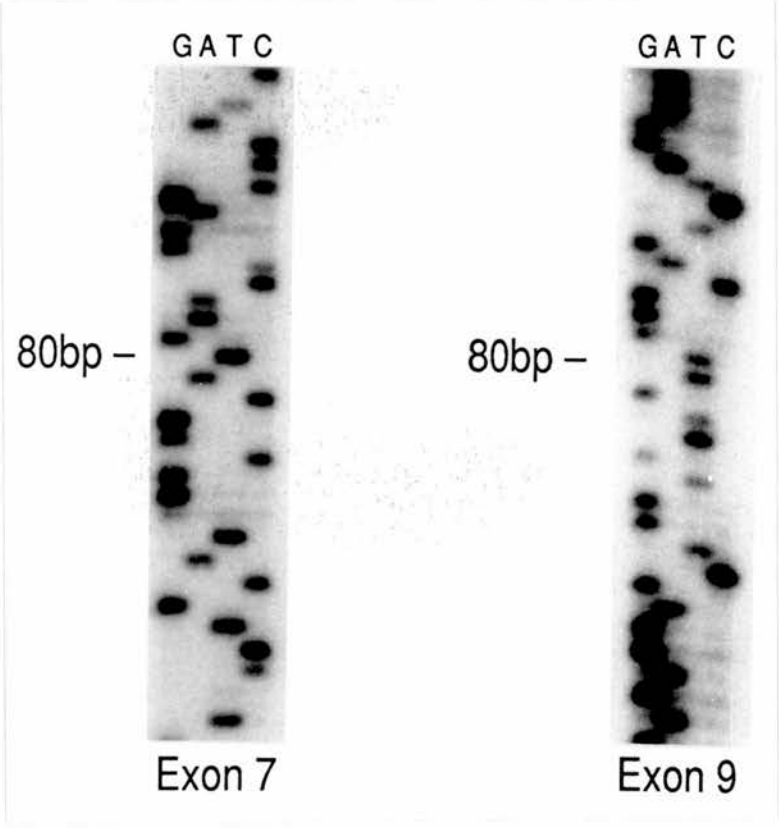


Figure 4.14 Sequence analysis of fragment IV of tumour 85. Arrows indicate the 80th base pair into fragment IV.



In order to determine whether any of the 25 mutations were uncharacterised polymorphisms or hereditary mutations, patient lymphocyte DNA was examined either by the HOT technique or by sequencing for the base change present in the tumour (Figures 4.15, 4.16). In all 25 cases the change in sequence was found only in the tumour DNA, confirming that the mutations were somatically acquired.

4.5 Sequence confirmation of p53 mutations by analysis of modified fragments.

Once the mutation was identified by sequencing, the fragment sizes produced by both HA and OsO_4 modification and piperidine cleavage were checked to see if they concurred with the expected fragment sizes. Unexpected bands can be explained by the modification of bases adjacent to the site of mismatch. Adjacent base pairs, though not mismatched themselves, can also be disrupted and are sometimes modified along with the mismatched bases (Cotton and Duncan Campbell 1989). Tables 4.2, 4.3 and 4.4 show the confirmatory cleaved bands observed with the p53 mutations and polymorphisms identified.

By comparing the mismatches detected by the HOT technique using either wild type or tumour probe DNA, it is apparent that although the HOT technique is able to detect some GT mismatches, eg the codon 213 polymorphism (Table 4.2), a number of GT mismatches are not detected by this technique, eg mutations in tumours 98 (Table 4.3), 101 and 110 (Table 4.4). The distortion of the double helix created by various mismatches is thought to depend on the bases involved. Mismatches such as CA and CC are thought to create the most distortion while GT mismatches are thought to cause the least (Cotton 1989). It is perhaps for this reason that the OsO_4 modification of mismatched thymine in GT mismatches is sometimes not sufficient for mutation detection.

Figure 4.15 Detection of mismatches in exons 7, 8 and 9 of the p53 gene in patient control DNA: HA modification and piperidine cleavage of heteroduplexes between wild type probe DNA and tumour target DNA. No cleaved fragments are detected. Tumour 25 (T25) was used as a positive control.

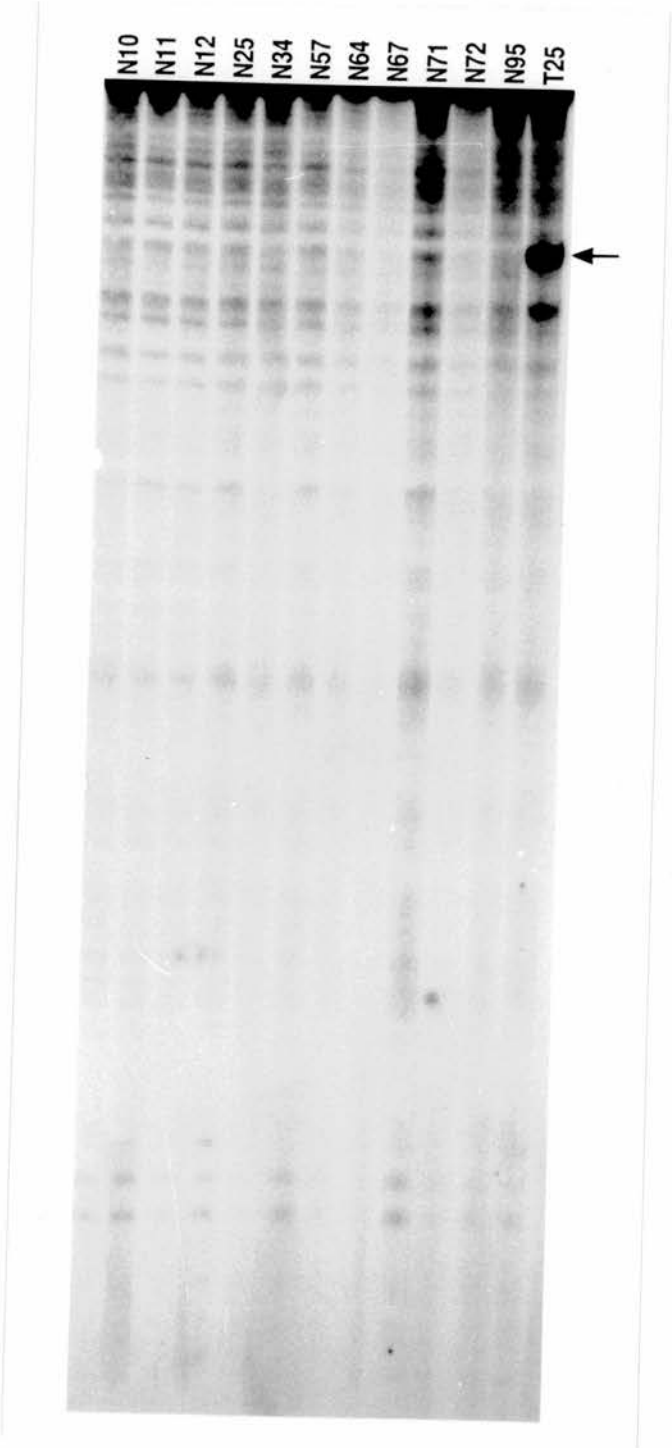
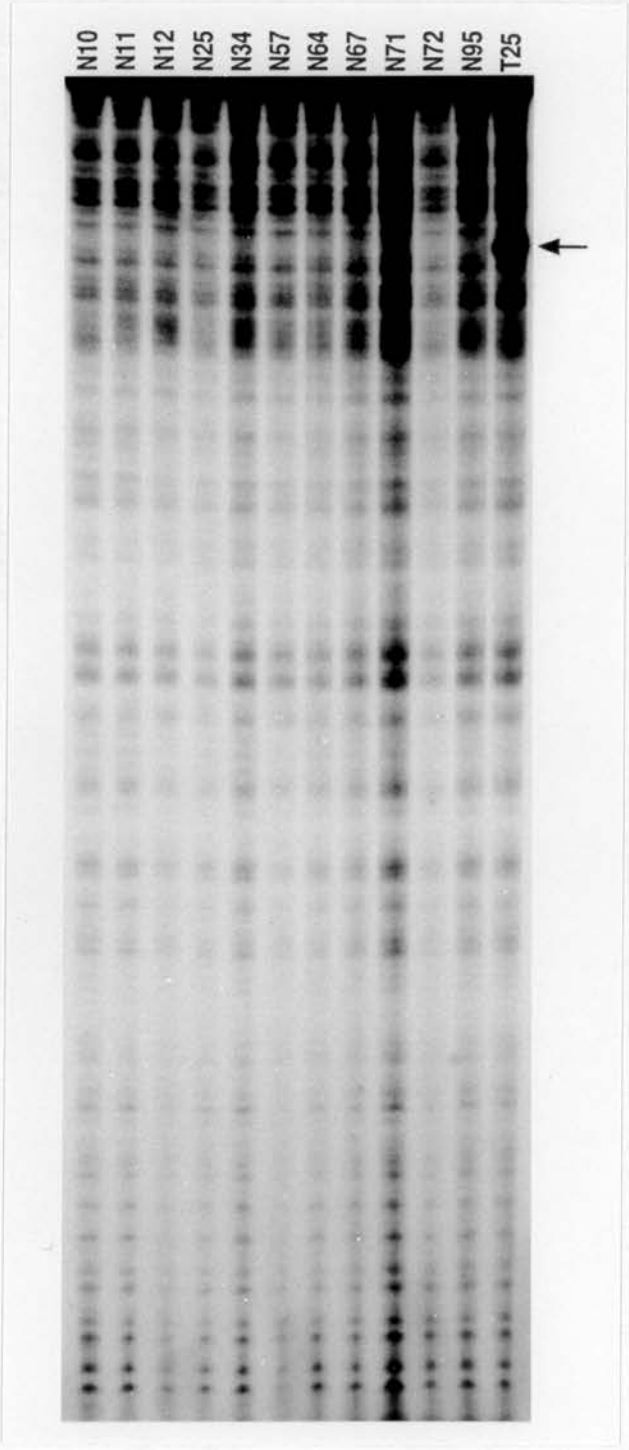


Figure 4.16 Detection of mismatches in exons 7, 8 and 9 of the p53 gene in patient control DNA: OsO_4 modification and piperidine cleavage of heteroduplexes between wild type probe DNA and tumour target DNA. No cleaved fragments are detected. Tumour 25 (T25) was used as a positive control.



Chapter 5

Analysis of p53 mutations in primary human breast cancer

5.1 Frequency of p53 mutations in primary breast cancer.

5.1.1 Estimating the incidence of p53 mutations in primary breast tumours.

Using the HOT technique 25 somatically acquired sequence alterations were detected in exons 5-9 of the p53 gene in 78 primary breast tumour samples. Twenty four of these alterations were identified by sequencing. The nucleotide and amino acid alterations together with LOH data are shown in Table 5.1.

Table 5.1 p53 mutation and LOH data in primary breast tumours.

Tumour Number	Codon	Nucleotide Change	Protein Change	BHp53 LOH	MCT35 LOH	YNZ22 LOH
8	208	GAC>GTC	Asp > Val	-	-	-
10	194	CTT>CGT	Leu > Arg	-	-	-
11	140	GAC>GΔC	Frameshift	-	-	-
18	179	CAT>TAT	His > Tyr	L	U	
21	192	CAG>TAG	Gln > Stop!	L	L	
25	186	GAT>TAT	Asp > Tyr	-	-	-
34	182	TGC>TGA	Cys > Stop!	U	L	N
57	175	CGC>CAC	Arg > His	L	L	-
64	213	CGA>TGA	Arg > Stop!	-	U	
67	163	TAC>AAC	Tyr > Asn	U	U	
71	141	TGC>TAC	Cys > Tyr	-	-	-
72	194	CTT>CGT	Leu > Arg	L	U	-
95	213	CGA>TGA	Arg > Stop!	U	U	
113	194	CTT>CGT	Leu > Arg	-	-	-
114	175	CGC>CAC	Arg > His	-	-	-
8	285	GAG>AAG	Glu > Lys	-	-	-
12	276	GCC>CCC	Ala > Pro	-	-	-
61	273	CGT>CAT	Arg > Leu	-	-	-
84	273	CGT>CTT	Arg > Leu	-	-	-
85	ND	ND	ND	N	-	-
88	237	ATG>ATT	Met > Ile	-	-	-
92	255	ATC>ΔΔΔ	Ile deletion	U	-	-
98	239	AAC > GAC	Asn > Asp	-	U	-
101	245	GGC>GAC	Gly > Asn	L	U	L
110	282	CGG>CTG	Arg > Leu	-	-	-

L - LOH
U - uninformative
N - No LOH
ND - Not determined
Δ - single base deletion

One of the tumour samples, number 8, contained two mutations, one in exon 6 and another in exon 8. For the purposes of estimating the frequency of breast tumours containing p53 mutations these two mutations effectively count as a single mutation. When estimating p53 mutation frequency from this data, a number of points must be considered. The estimate will be affected by the efficiency of the HOT mutation detection technique, the incidence of mutations occurring at sites other than in exons 5-9 of the p53 gene and, because of the design of the primers used, the inability of the technique to detect mutations at a number of bases in exons 5, 6, 7 and 9. These will be discussed in the following section.

5.1.2 Factors effecting the estimation of p53 mutation frequency in breast tumours.

Non-sequencing mutation detection techniques are now widely used, although little work has been done on the relative efficiencies of the different techniques. Theophilus *et al.* (1989) compared the use of RNase A protection, chemical cleavage and DGGE techniques and concluded that DGGE was the most reliable and informative method. In a recent study by Condie *et al.* (1993), a direct comparison was made between the three techniques most commonly used to detect p53 mutations in breast tumour samples, SSCP, CDGE and HOT (Prosser *et al.*, 1990; Borresen *et al.*,1991; Mazars *et al.*, 1992b; Osborne *et al.*,1991; Runnebaum *et al.*, 1991; Thompson *et al.*, 1992). The efficiency of each technique was determined by the analysis of DNA samples containing known p53 mutations. Both SSCP and CDGE techniques were found to detect 90% of all p53 mutations. The HOT technique, when using both wild type probe DNA with mutant target DNA and mutant probe DNA with wild type target DNA, had a detection efficiency of 100%. In this way any GT mismatches missed using one type of probe DNA would be detected through a CA mismatch when using the other type.

The HOT technique was carried out using both wild type and tumour probe DNA to detect p53 mutations in exons 7-9 in this study (section 4.4). However only wild type probe DNA with tumour target DNA fragments were used to detect p53 mutations in exons 5 and 6. Theoretically therefore AT>GC substitutions occurring in this region may not be detected and indeed, no AT>GC mutations were detected in exons 5-6 (Table 5.1). By analysing the mutations identified by other

detection techniques of p53 in breast cancer it can be seen that 3/20 (15%) of mutations in exons 5-6 are AT>GC substitutions (section 5.3.5). Therefore, the 15 mutations found in exons 5 and 6 of the p53 gene could represent an actual incidence of 18 mutations, giving a total of 27 mutations in exons 5-9.

Although the majority of mutations found in the p53 gene have been found within exon 5-9, a significant number of mutations have been identified outside this region. In a study of compiled p53 alterations from a wide variety of cancers, 22 out of 368 mutations (6%) were recorded outside exons 5-9, in exons 1-4, 10 and 11, and in introns 3, 4, 5, 6, 7 and 9 (Caron de Fromentel and Soussi 1992). This frequency is probably an underestimate due to the heavy bias in the majority of studies towards mutation detection in exons 5-8 or 5-9 alone. However no base changes were detected in this study in introns 5, 7 and 8 which were included in the PCR fragments screened by the HOT technique. No mutations were detected in exon 4 in 15 (Chen *et al.*, 1991a) and 24 (Osborne *et al.*, 1991) breast tumours or in exon 2 in 96 breast tumours (Mazars *et al.*, 1992b). Five of 59 (8%) breast tumours analysed by multiplex PCR showed aberrant bands in exons 2-4 and 10 and 11 (Runnebaum *et al.*, 1991). If a figure of 10% is allowed for mutations occurring outside exons 5-9 then the 27 mutations in exons 5-9 would represent 30 mutations in the entire gene.

Ideally, the oligonucleotide primers used to amplify exons 5-6 and 7-9 should be located within the intron sequences flanking the exons. Due to the incorporation of the primers into the fragment during amplification any base change occurring within the primer sequence would not be detected. Due to the lack of information on the intron sequences of the p53 gene at the time of the oligonucleotide primer design, it was necessary to include five oligonucleotide bases at the beginning of exons 5 and 7 and at the end of exons 6 and 9 in the primer sequences. Therefore any mutations occurring in codons 126, 224, 225 and 310, in the first two bases of codons 127 and 226 or in the last two bases of codons 223 and 311 would not be detected. However, other studies detecting p53 mutations in breast tumour samples have not identified mutations occurring at any of these sites. Indeed mutations at these codons have not been identified in any type of tumour sample (Hollstein *et al.*, 1991a; Caron de Fromentel *et al.*, 1992). Therefore the estimate of p53 frequency has not been modified to allow for potential mutations at these sites.

After allowing for mutations outside exons 5-9 and the mutation detection efficiency of the HOT technique the 24 primary breast tumours containing p53 mutations would represent an estimated incidence of 38% (30/78). Table 5.2 compares this figure with published data from other studies on p53 mutation frequency in breast tumours. Due to differing approaches of analysis the different sets of data have been modified in order to allow direct comparison. The estimated incidence of p53 mutation range from 16 to 46% although most of the estimates fall between 35% and 46%. On the strength of these figures it is estimated that approximately 40% of primary breast tumours contain a p53 mutation.

5.1.3 Immunohistochemical estimates of p53 mutation frequency in breast tumours.

The p53 mutation frequency in breast tumours has been estimated on the basis of immunohistochemical analysis, involving the staining of tumour tissue sections with p53 specific antibodies in order to detect over-expression of the p53 protein. Due to the short half life and low level of expression of the wild type p53 protein in normal cells, the p53 protein is virtually undetectable by immunohistochemical methods. p53 mutation can result in a stable protein with an increased half life, resulting in expression at a level detectable by immunohistochemistry. Thus any cell staining with p53 specific antibodies may indicate the expression of a mutant form of p53.

Estimation of p53 mutation frequency in primary breast tumours by immunohistochemical detection has been attempted by many groups (Table 5.3). Comparison of 20 immunohistochemical studies reveals estimates ranging between 14% (41/289) and 67% (18/27) (Isobe *et al.*, 1992; Moll *et al.*, 1992). Variation between studies, discussed below, could be due in part to the type and condition of the sample used, the use of different antibodies detecting different p53 epitopes and varying criteria used for the definition of a positively staining sample.

The study by Bartek *et al.* (1990b) detected antibody staining in 56% of frozen samples while only 20% of paraffin embedded tissue samples showed positive staining with the same antibodies. Chang *et al.* (1991) observed that 58% of breast tumour samples stained with PAb1801 while only 21% stained with PAb421 (Table 5.3). The majority of studies reveal different expression patterns of the p53 protein and have been unable to classify tumours into positive or negative

Table 5.2 Frequency of breast tumours with mutations in the p53 gene (DNA based methods of detection)

Method (a)	Tumours Examined	Tumours with mutation	Total number of tumours with mutation (c)	Exons studied	Tumours with mutation in exons 5-9	Tumours with mutation in entire gene (e)	Estimated frequency of tumours with mutations in the entire gene (%)	Reference
CDGE	32	10	11	5,7,8	13 (d)	14	43	Borresen *et al.*, 1991
HOT	78	24	27	5 -9	27	30	38	This study
SSCP (b)	24	9	10	5 - 8	10 (d)	11	46	Osborne *et al.*, 1991
SSCP (b)	59	10	11	5 - 9	11	12	20	Runnebaum *et al.*, 1991
SSCP	96	17	19	2, 5 - 9	19	21	22	Mazars *et al.*, 1992b
PCR sequencing	11	4	4	5 - 9	4	4	36	Kovach *et al.*, 1991
PCR sequencing	44	14	16	5 - 9	16	18	41	Sommer *et al.*, 1992
RNase protection	26	3	6	All exons	-	6	23	Osborne *et al.*, 1991
Multiplex PCR (b)	59	21	-	2 - 11	-	21	36	Runnebaum *et al.*, 1991

(a) Most of these techniques need to be used in conjunction with sequencing to avoid false positives caused by polymorphisms or neutral mutations.
(b) Not sequenced.
(c) Total number of tumours with mutation has been estimated considering the following detection efficiencies of the various techniques used: CDGE 90%; SSCP 90%; PCR sequencing 100%; HOT exons 5-6, 90%; HOT exons 7-9, 100% (Section 5.2); RNase Protection assay 50% (Chiba *et al.*, 1990)
(d) Data on all p53 mutations identified in breast tumours was used to estimate the frequency of mutations present in exons 6 and 9.
(e) p53 mutations outside of exons 5-9 have been estimated to account for 10% of the mutation in the entire gene.

Table 5.3 Frequency of breast tumours with mutations in the p53 gene. (Immunohistochemical methods of detection).

Method	Number of tumours examined	Number of tumours with mutations	Mutation frequency (%)	Reference
PAb421 PAb1801 on frozen and paraffin embedded sections	200 88	31 40	15.5 45.5	Cattoretti *et al.*, 1988
PAb 1801, PAb240,.PAb421 on frozen sections	81	45	56	Bartek *et al.*, 1990b
PAb 1801, PAb240, PAb421 on paraffin embedded sections	94	19	20	Bartek *et al.*, 1990b
CM - 1 on paraffin embedded sections	42	26	62	Bartek *et al.*, 1991
PAb 1801, PAb240, PAb 421 on frozen sections	49	11	22	Davidoff *et al.*, 1991a
PAb1801, PAb240 on frozen sections	73	42 - 48	58 - 66	Varley *et al.*, 1991
PAb240 on frozen and paraffin sections	111	59	53	Horack *et al.*, 1991
PAb240, C19, PAb1801, JG8 and PAb421 on frozen sections	80	44	55	Walker *et al.*, 1991
PAb421 PAb1801 PAb240 on frozen sections	19 19 19	4 11 7	21 58 37	Chang *et al.*, 1991
PAb1801 on frozen sections	184	50	27	Davidoff *et al.*, 1991c
PAb 1801 on frozen sections	90	32	36	Ostrowski *et al.*, 1991
PAb421 on FNA smears	37	19	51	Koutselini *et al.*, 1991
OM-11-918 on frozen sections	32	6	19	Borresen *et al.*, 1991
PAb 1801 on frozen sections	44	13	30	Thompson *et al.*, 1992
PAb 1801 on formalin fixed sections	146	62	43	Poller *et al.*, 1992

Table 5.3 (continued)

Method	Number of tumours examined	Number of tumours with mutations	Mutation frequency (%)	Reference
PAb 1801 on frozen sections	27	18	67	Moll *et al.*, 1992
PAb1801 on methacarn fixed samples	46	17	37	Porter *et al.*, 1992a
CM-1 on formalin fixed sections	289	41	14	Isola *et al.*, 1992
PAb1801 on paraffin embedded and frozen sections	498	110	22	Thor *et al.*, 1992
PAb 1801 and PAb240 on frozen samples	700	362	52	Allred *et al.*, 1993

staining groups (Bartek *et al.*, 1990b; Bartek *et al.*, 1991; Chang *et al.*, 1991; Davidoff *et al.*, 1991c; Midgley *et al.*, 1992; Moll *et al.*, 1992; Porter *et al.*, 1992a). Most studies identify three or four basic patterns: intense nuclear staining in the majority of tumour cells; variable staining in only a proportion of tumour cells ranging from 90% to single isolated cells or cell clusters; cytoplasmic staining; and absence of cell staining. Consequently the criteria for defining a positively staining tumour vary widely. For example, those tumours showing antibody staining in any cell are scored as positive by Thor *et al.* (1992) while only those showing staining in greater than 20% of cells are scored positive by Isola *et al.* (1992). Similarly cytoplasmic staining is counted as positive in studies by Horak *et al.* (1991) and Moll *et al.* (1992) but not in those by Poller *et al.* (1992) and Varley *et al.* (1991).

In spite of this, several of the studies detect over-expression of p53 protein in 50-66% of breast tumour samples, a figure 10-20% in excess of the mutation frequency observed at the DNA level (Bartek *et al.*, 1990b; Varley *et al.*, 1991; Horak *et al.*,1991; Walker *et al.*, 1991; Chang *et al.*, 1991; Koutselini *et al.*, 1991; Moll *et al.*, 1992; Allred *et al.*, 1993). This may be due to an over estimated efficiency of mutation detection by DNA based techniques or a greater than expected number of mutations occurring at sites other than exons 5-9.

Alternatively, there is evidence to suggest that over-expression of the p53 protein may not necessarily indicate the presence of a mutated p53 gene. p53 protein levels are known to be affected by factors such as differentiation state (Linzer and Levine 1979; Reich *et al.*, 1983), cell cycle progression (Reich and Levine 1983) and complex formation with other proteins such as the SV40 T antigen (Lane and Crawford 1979). High levels of apparently wild type p53 have been found to be expressed by 4 neuroblastoma derived cell lines (Davidoff *et al.*, 1992b). A high level of p53 protein has been found in most epithelial and mesenchymal cells from two individuals who do not have a constitutional p53 mutation and an alteration in the degradation of the p53 protein has been suggested as a possible mechanism (Barnes *et al.*, 1992). Evidence for the expression of wild type protein in a mutant configuration has been found in a number of studies (Gamble and Milner 1988; Milner and Watson 1990; Cook and Milner 1990). Taken together, these observations suggest that

over-expression of the p53 protein or reactivity with a mutant specific antibody such as pAb240 may not be indicative of a mutated p53 gene.

Despite this evidence, data from several studies have indicated a close correlation between p53 over-expression and p53 mutation. Of 43 positively staining breast tumours and cell lines, 39 have been shown to possess mutations in the p53 gene (Bartek *et al.*, 1990a; Davidoff *et al.*, 1990; Davidoff *et al.*, 1991a; Varley *et al.*, 1991; Borresen *et al.*, 1991; Sommer *et al.*, 1992; Thor *et al.*, 1992). A close correlation has also been reported in lung, colon, ovary and oesophagus tumours and cell lines (Bennet *et al.*, 1991; Iggo *et al.*, 1990; Rodrigues *et al.*, 1990; Marks *et al.*, 1991; Gusterson *et al.*, 1991). However, no correlation was found between p53 mutation and p53 over-expression in breast tumour samples in a study by Thompson *et al.* (1992). In one study tumour samples were split into either nuclear or cytoplasmic staining groups (Moll *et al.*, 1992) and p53 mutations were associated with tumour samples showing nuclear staining. Antibody staining therefore could be indicative of a p53 mutation in only a specific subset of tumours. Furthermore the two distinct patterns of p53 expression suggested cytoplasmic exclusion as a mechanism for p53 protein inactivation. In a comparison of antibody staining and mutation detection by SSCP in exons 5-9 in 171 breast tumours concordance between the two tests was found in only 108 (62%) samples (Allred *et al.*, 1993). Of the remaining 63 discordant cases, 58 were SSCP negative and immunohistochemically positive and 5 were immunohistochemically negative and SSCP positive. Even after considering the possibility of mutations occurring outside exons 5-9 and the less than 100% detection rate of the SSCP technique these results strongly suggest that over-expression of the p53 protein can occur without mutation of the p53 gene.

In addition, there are reasons to suggest that the immunohistochemical methods would not detect all mutations. For example, the majority of p53 genes with stop codons truncating the protein before codon 370 would go undetected by the antibody Ab421, which detects an epitope between the amino acids 370 and 378, and indeed p53 mutations have been found in negatively staining breast tumour material in several studies (Bartek *et al.*, 1991; Borresen *et al.*, 1991; Sommer *et al.*, 1992; Thompson *et al.*, 1992).

Overall, while immunohistochemical analysis provides some indication on how frequently the p53 protein is over-expressed in tumours it cannot, for several reasons, be relied upon to determine the p53 mutation frequency. Until a large study of mutations detected at both the protein and DNA sequence levels is undertaken, no conclusions can be drawn about the mutation detection efficiency of immunohistochemical analysis.

5.1.4 Incidence of p53 constitutional mutations in sporadic breast cancer patients.

Following the identification of p53 mutations in Li-Fraumeni syndrome (LFS) patients the possibility that constitutional p53 mutations could be involved in other hereditary cancer syndromes or a proportion of sporadic cancers was proposed (Marx 1990; Vogelstein 1990). Screening for constitutional p53 mutations focused on patients with breast and adrenocortical carcinomas, sarcomas and leukemias since these tumour types figure predominantly in the LFS cancer spectrum. Several investigations selected patients showing early onset of cancer or multiple tumours, features characteristic of hereditary disease, in the hope of increasing the chances of detecting a p53 mutation.

A mutation frequency of 6.8% (4/59) was determined in children and young adults with second cancers (Malkin *et al.*, 1992). Two p53 mutations were found in two families from a cohort of patients with childhood adrenocortical carcinoma (Sameshima *et al.*, 1992a). Two constitutional p53 mutations in two LFS families were detected following analysis of the p53 gene in 17 osteosarcoma patients (Porter *et al.*, 1992b). Four of 25 (16%) paediatric lymphoblastic leukemia patients were found to possess a constitutional p53 mutation with LFS indicated in only one of the kindreds (Felix *et al.*, 1992a). Similarly, a total of 8 constitutional p53 mutations were detected in a set of 196 (4%) sarcoma patients. Five of these mutations originated in 15 patients having multiple tumours or a family history of cancer. Only one of the patients had a family history fully consistent with the LFS in its strictest definition (Toguchida *et al.*, 1992). Both these studies indicate that a high proportion of non-LFS familial sarcoma patients (4/15, 27%) and non-LFS paediatric lymphoblastic leukemia patients (12%) possess a constitutional p53 mutant allele.

It is possible that a small subset of sporadic breast cancers patients (estimated to be less than 5%) and a proportion of HBC families may possess constitutional p53 mutations (Vogelstein 1990). A total of 30 HBC families have been screened for p53 mutations in two studies (Prosser *et al.*, 1992; Warren *et al.*, 1992) with neither detecting any constitutional p53 mutation. In addition, 30 women with at least one first degree relative with breast cancer also had no detectable p53 mutation (Borresen *et al.*, 1992). One study detected no constitutional p53 mutations in 126 patients who had developed breast cancer before the age of 40 (Sidransky *et al.*, 1992a), while a similar study by Borresen *et al.* (1992) detected one p53 mutation in a set of 40 women who had developed breast cancer before the age of 35. No mutations were found in patients selected on the basis of bilateral breast cancer (Lidereau and Soussi 1992). One of 167 (0.6%) sporadic breast tumour patients was found to possess the mutation CGC > CAC at codon 181, resulting in an amino acid change of Arg > His (Borresen *et al.*, 1992). However since the mutant p53 protein has been shown to possess wild type p53 properties when compared with other constitutional p53 mutant proteins (Frebourg *et al.*, 1992) it has been suggested that it is functionally silent and may impart no increased cancer risk (Borresen *et al.*, 1992).

In this study twenty five mutations were identified in 78 sporadic primary breast tumours from 73 patients using the HOT technique. No mutation occurring in any of the 25 tumours was detected in the corresponding lymphocyte DNA as described in section 4.4. A similar study from another series of Scottish patients detected a constitutional p53 mutation in 1/60 sporadic breast cancer patients (Thompson *et al.*, 1992). The mutation was a GC>AT substitution at the second nucleotide of codon 267 and occurred in a non-LFS cancer family. When both sets of data are combined, the constitutional p53 mutation frequency in sporadic breast cancer patients is estimated as 1/133 (0.7%) (Prosser *et al.*, 1992), a similar figure to that found by Borresen *et al.* (1992). These series of experiments indicate that constitutional p53 mutation is a rare etiological factor in sporadic breast cancer.

5.2 Relationship between p53 mutation and LOH on chromosome 17p in primary breast cancer.

5.2.1 p53 mutation and allelic loss in primary breast cancer.

The p53 gene has been shown to possess the characteristics of both oncogene and tumour suppressor gene (section 1.5.6). Data on p53 mutation and LOH enable speculation on the state of the two p53 alleles and suggest mechanisms by which the p53 gene may contribute to the maintenance of a malignant phenotype. If the p53 gene is simply acting as a tumour suppressor then, in accordance with Knudson's two hit hypothesis, p53 mutation should frequently be accompanied by LOH, indicating the inactivation of both copies of the gene. If the mutant p53 gene is behaving as a dominant oncogene then p53 mutation need not be accompanied by the loss of the second wild type allele.

Various studies have attempted to correlate the LOH on chromosome 17p and p53 mutation in breast cancer (Nigro *et al.*, 1989; Prosser *et al.*, 1990; Borresen *et al.*, 1991; Chen *et al.*, 1991a; Davidoff *et al.*, 1991a; Osborne *et al.*, 1991; Varley *et al.*, 1991; Thompson *et al.*, 1992). However care must be taken when using LOH data as an indicator for the loss of the wild type p53 allele in tumour samples. Evidence implicating two independent areas of loss on chromosome 17p has been found in a number of malignancies. One is at 17p13.1, the site of the p53 gene, and the other is more telomeric at 17p13.3 (Section 3.9). Thus, unless the pattern of allele loss on chromosome 17p determines a single locus, as has been shown in colon cancer (Baker *et al.*, 1989), LOH at the p53 locus cannot be predicted by LOH at the more telomeric site, eg when using the probe YNZ22 (Nigro *et al.*, 1989; Chen *et al.*, 1991a; Varley *et al.*, 1991).

5.2.2 Association of p53 mutation with LOH.

There are 30 tumours in this study which are informative at the BHP53/TP53 or MCT35/D17S31 loci, which map near the p53 gene at 17p13.1, and have been screened for p53 mutations in exons 5-9 using the HOT technique. The precise codon changes and LOH data in tumours with a p53 mutation are shown in table 5.1. Six of the 7 (86%) tumours (tumours 18, 21, 34,

57, 72 and 101) which possess a p53 mutation in exons 5-9 show LOH at nearby loci. Other breast cancer studies report that p53 mutations are accompanied by LOH, at loci near the p53 gene, at a high frequency: 100% (10/10) (Borresen *et al.*, 1991); 57% (4/7) (Davidoff *et al.*, 1991a); 56% (5/9) (Osborne *et al.*, 1991); and 75% (9/12) (Thompson *et al.*, 1992).

In addition to determining LOH at the p53 gene locus, several investigators have directly determined the presence or absence of the wild type allele using sequence data and SSCP autoradiographs. Using these methods, a high frequency of loss is also observed in breast tumours and cell lines: 100% (8/8) (Runnebaum *et al.*, 1991); 100% (4/4) (Osborne *et al.*, 1991); 100% (2/2) (Kovach *et al.*, 1991); 57% (4/7) (Davidoff *et al.*, 1991a); and 93% (13/14) (Sommer *et al.*, 1992). A similar high frequency of homozygous and hemizygous mutations has been detected in tumours of the ovary, oesophagus, brain, liver, uterus, gut, as well as in leukemia (Gaidano *et al.*, 1991; Hensel *et al.*, 1991; Hsu *et al.*, 1991; Mashiyama *et al.*, 1991; Okamoto *et al.*, 1991a; Okamoto *et al.*, 1991b; Shaw *et al.*, 1991; Tamura *et al.*, 1991).

In this study 24 p53 mutations have been characterised. Direct comparison between the intensities of the mutant and wild type sequencing bands showed that the mutant allele was at least twice the intensity of the wild type allele in 11 (46%) tumours, suggesting that the p53 mutation was accompanied by loss of the wild type copy of the p53 gene. However, in the remaining 13 (54%) mutations the wild type sequencing band was either equal to or greater than the intensity of the mutant sequencing band. While these may reflect genuine p53 mutations without loss of the wild type allele, the relative intensities of the mutant and wild type bands may simply be due to the contamination of the tumour sample by normal tissue or the presence of tumour heterogeneity. This problem is highlighted in a study by Mazars *et al.* (1992b) which found that only 2/16 breast tumours with p53 mutations were accompanied by loss of the wild type allele. Previous data suggest that the breast tumour samples used in this present study are frequently contaminated with normal tissue or show tumour heterogeneity (section 3.9) and could explain the low frequency of loss observed when analysing the mutations using sequence data.

5.2.3 p53 mutation without LOH

The loss of the wild type allele in the majority of breast tumour samples with a p53 mutation indicates the importance of the removal of the tumour suppressing function of wild type p53 in breast cancer development. However one of the 7 (14%) tumours with a p53 mutation showed no accompanying LOH (tumour 85). A number of similar tumours have been identified in other breast cancer studies: 3/7, 43% (Davidoff *et al.*, 1991a); 4/9, 44% (Osborne *et al.* 1991); 1/14, 7% (Sommer *et al.*, 1992), 3/12, 25% (Thompson *et al.*, 1992). These tumours show no LOH at the p53 locus and so suggest that the mutant p53 gene is acting as an dominant oncogene. However, the remaining allele may be inactivated by mechanisms other than mutation in exons 5-9 and deletion (section 5.2.4). Alternatively these tumours may simply represent a stage following p53 mutation and before allele loss, as has been suggested in colon carcinomas (Baker *et al.*, 1990a).

Certain mutant p53 genes are known to be able to act as oncogenes (Hinds *et al.*, 1990; Halevy *et al.*, 1990; Milner and Metcalf 1991) and may influence tumorigenesis in a dominant manner. An interesting hypothesis would be that while weakly or non-oncogenic p53 mutants require loss of the remaining wild type allele, strongly oncogenic mutations would require no loss to effect a growth advantage. Although p53 mutants are known to possess different properties the relative oncogenicity of only a few has been determined. Hinds *et al.* (1990) and Halevy *et al.* (1990) have compared p53 mutations on the basis of their ability to transform primary rat cells in culture while Milner and Metcalf (1991) have compared mutant proteins for their ability to drive wild type p53 protein into the mutant conformation. From these three studies, a number of p53 mutations have been categorised as either strongly oncogenic (132 Phe, 135 Val, 151 Ser, 175 His, 247 Ile and 273 Pro) or weakly oncogenic (248 Trp, 270 Cys, 273 His and 281 Gly).

Only one breast tumour sample in this study possessed any mutation of measured oncogenicity. Tumour 57 possesses the strongly oncogenic 175 His mutation and so might be expected to show retention of the normal wild type allele. This tumour however shows LOH at the MCT35/D17S31 locus and sequencing of this mutation shows a near complete absence of the wild type allele (Figure 4.12). Other breast cancer studies identifying tumours containing p53 mutations of known oncogenicity show either LOH or loss of the wild type allele upon sequencing regardless of

the proposed strength of the mutation (Nigro *et al.*, 1989; Borresen *et al.*, 1991; Chen *et al.*, 1991a; Kovach *et al.*, 1991; Varley *et al.*, 1991; Thompson *et al.*, 1992). The oncogenic properties of the various p53 proteins therefore do not seem to determine the loss of the remaining allele in breast tumours.

5.2.4 LOH without p53 mutation.

LOH at the p53 locus without apparent alteration in the remaining p53 allele has been determined in a proportion of samples. Of 16 tumours in this series with LOH near the p53 locus, 10 (63%) apparently have wild type p53 DNA only, suggesting that many tumours show loss of a p53 allele without mutation of the remaining copy. The occurrence of LOH without apparent mutation has been identified in breast tumours (Davidoff *et al.*, 1991a; Osborne *et al.*, 1991; Sommer *et al.*, 1992; Thompson *et al.*, 1992) and in other malignancies (Baker *et al.*, 1990a; Okamoto *et al.*, 1991a,b: Frankel *et al.*, 1992; Miller *et al.*, 1992; Seruca *et al.*, 1992) and several possible explanations have been suggested.

The alteration of the remaining wild type p53 can potentially occur at the DNA and protein levels via mechanisms other than mutation in exons 5-9 and deletion. These include rearrangement (Mowat *et al.*, 1985), promoter mutation (Rovinski *et al.*, 1987; Bookstein *et al.*, 1990a), altered transcription (Mulligan *et al.*, 1990; Thompson *et al.*, 1990), inactivation of the wild type protein by mutant forms (Milner and Metcalf 1991), altered conformation (Cook and Milner 1990; Milner and Watson 1990), altered degradation (Barnes *et al.*, 1992), hypermethylation (Sakai *et al.*, 1991), alteration of intronic sequences (Beenken *et al.*, 1991), localisation to the cytoplasm (Shaulsky *et al.*, 1991; Moll *et al.*, 1992) and binding of the p53 protein to cellular proteins such as the murine double minute 2 (MDM2) protein (Oliner *et al.*, 1992; Momand *et al.*, 1992) and viral proteins (Lane and Crawford 1979). None of these types of alteration would be detected in either the LOH or p53 mutation analyses.

Loss or deletion of chromosomes are thought to occur randomly in the tumorigenic process, with those changes conferring a growth advantage being selected. However LOH on 17p in a proportion of tumours may represent a random or non-selected loss, an alteration that provides no

selective advantage or disadvantage to the clone. Another possibility is that the mutation detection technique used may not have detected the corresponding p53 mutation. This is especially relevant were the mutation techniques do not detect 100% of the mutations eg SSCP and CDGE both have a detection rate of 90%. The mutation may have occurred outside the regions screened as frequently only exons 5-8 or 5-9 are screened for the presence of mutations. There may be a selective advantage resulting in the deletion of only one copy of the p53 gene although this mechanism does not conform to the pattern expected of either a tumour suppressor gene or an oncogene (Bishop 1990). Finally, the allele loss at the p53 locus may involve a second tumour suppressor gene. Since LOH on chromosome 17p at the p53 locus frequently involves 17p13.3 loci and occasionally whole chromosome loss (Andersen *et al.*, 1992) it may simply reflect the inactivation of other tumour suppressor genes on the long or short arms of chromosome 17 (Osborne *et al.*, 1991).

5.2.6 Discussion

High frequencies of LOH on chromosome 17p and p53 gene mutation were initially detected in colon carcinomas and are thought to play integral parts in colon tumorigenesis (Vogelstein *et al.*, 1988; Baker *et al.*, 1990a). Following these studies many groups have analysed a wide range of tumour types for similar alterations. The main aim of the series of experiments described in sections 5.1 and 5.2 was to determine the prevalence, and therefore the potential importance, of these genetic abnormalities in primary human breast cancer. LOH on chromosome 17 at loci near the p53 gene and p53 gene mutation are estimated to occur in 49% and 40% of breast tumour samples respectively. These figures suggest that alteration of the p53 gene is not as frequent, and therefore not as important, in breast tumorigenesis as it is in the development of other common solid tumours. Colon carcinomas show a frequency of LOH on chromosome 17p of 64-76% and a frequency of p53 gene mutation of 64-71% (Vogelstein *et al.*, 1988; Baker *et al.*, 1990a; Shaw *et al.*, 1991; Cunningham *et al.*, 1992). LOH on chromosome 17p and p53 gene mutation are observed in 88-100% and 45-85% of lung cancer samples respectively (Yokota *et al.*, 1987; Weston *et al.*, 1989; Chiba *et al.*, 1990; Hensel *et al.*, 1991; D'Amico *et al.*, 1992; Miller *et al.*, 1992; Sameshima *et al.*, 1992b; Tsuchiya *et al.*, 1992). These observations indicate that the

alteration of the p53 gene is an important step in colon tumorigenesis and perhaps an essential one in the development of lung cancer.

However, the results of the current study in breast tumours can only indicate the importance of the alteration of the p53 gene by the mechanisms of mutation and allelic loss. Alteration of wild type p53 can potentially occur at the DNA and protein levels via many different mechanisms (section 5.2.4). Studies in most types of cancer have concentrated on the mutation and loss of the p53 gene and have barely investigated the role of many other potential mechanisms of alteration. For example it is thought that the p53 protein is often inactivated in sarcomas through binding with the MDM2 protein, possibly as a result of amplification of the MDM2 gene (Oliner *et al.*, 1992; Vogelstein 1992). Similarly the binding of the viral protein E6 to the p53 protein, resulting in p53 protein degradation, has been found in HPV (human papilloma virus) associated cervical tumours (Schneffner et al., 1990). The p53 gene could therefore be altered in many breast tumours by mechanisms not yet investigated. The higher frequency of p53 protein alteration found in some p53 antibody staining studies compared to those using DNA based methods of mutation detection might suggest other mechanisms of alteration, such as p53 protein inactivation by complexing with other proteins or by a change in conformation, which could lead to the loss of the tumour suppressor function of the p53 protein. Further studies investigating p53 at the DNA, mRNA and protein levels should give a greater insight into the mechanisms by which p53 contributes to a malignant phenotype in breast tumours.

Overall, p53 mutations found in breast tumour samples are frequently accompanied by loss of the wild type allele suggesting a role as a tumour suppressor gene in the majority of breast tumours. While there is evidence to suggest that the p53 acts as an oncogene in a small proportion of breast tumours, the identification of tumour samples with p53 mutations and no concurrent LOH many simply reflect either the composition of the tumour material or the inactivation of p53 by mechanisms other than LOH.

5.3 p53 mutation analysis in breast cancer.

5.3.1 Mutagenesis of the p53 gene.

Mutations in the growth regulating proto-oncogenes and tumour suppressor genes are critical steps in tumorigenesis. Both internal (endogenous) and external (exogenous) factors are thought to play a part in the mutation of these genes. Endogenous factors include damage caused by free oxygen radicals (Lutz *et al.*, 1990), deamination of 5-methylcytosine to thymine (Ehrlich *et al.*, 1990), DNA depurination and DNA polymerase infidelity (Loeb and Cheng 1990). Exogenous factors include environmental carcinogens, many of which possess mutagenic properties, such as N-nitrosamines, polycyclic aromatic hydrocarbons and fungal toxins (Zeiger 1987).

Studies using prokaryotic and simple eukaryotic systems have shown that carcinogenic agents can show specificity for both the type and the location of the DNA mutation they induce (Skandalis and Glickman 1990). This has been demonstrated in mammals by the work done on the mutation of the ras oncogene by chemical carcinogens (reviewed in Harris 1991). Chemical treatment of test animals leads to a carcinogen-specific pattern of mutation of the ras gene in resulting tumours. For example, in a study by Zarbl *et al.* (1985) the ras mutations detected in the tumours of rodents exposed to methylating N-nitroso-N-methylurea were all GC>AT base substitutions, a result of the methylation of deoxyguanine followed by the mispairing of thymine during DNA synthesis. Although there are several deoxyguanine residues that would produce a transforming ras protein, all the mutations were located in the second nucleotide base of codon 35. This carcinogen-specific alteration of a defined DNA sequence establishes a direct link between the tumorigenic process and the influence of exogenous carcinogens.

Theoretically, therefore, the type of mutations observed in cancer cells can be linked to specific mechanisms of mutation. Analysing the type and locations of mutation identified in tumours may offer insight into the origins of the mutations (Vogelstein and Kinzler 1992). While many of the investigations of this type have concentrated on the ras oncogene, their use in implicating specific mutation mechanisms in tumours is limited due to the low mutation frequency of ras in the majority of human solid tumours and the limited number of codons which can be mutated to give an oncogenic form of the protein (Bos *et al.*, 1989). In contrast the p53 gene is ideally suited for this

type of analysis. p53 mutations can occur at many sites within the gene (Hollstein *et al.*, 1991a, Caron de Fromentel and Soussi 1992), thus potentially producing more informative mutation spectra, and are detected in many different tumour types, often at high frequencies (Baker *et al.*, 1990a; Chiba *et al.*, 1990; Hollstein *et al.*, 1991b; Mazars *et al.*, 1991; Sidransky *et al.*, 1991; Tamura *et al.*, 1991; D'Amico *et al.*, 1992; Frankel *et al.*, 1992).

5.3.2 Mutation spectra of the p53 gene in solid tumours.

The mutation spectrum of the p53 gene has been determined in two studies using p53 mutations detected in a range of malignancies (Hollstein *et al.*, 1991a; Caron de Fromentel and Soussi 1992). Mutations were found in over 100 of the 393 codons of the gene, with the majority located in exons 5-8. The mutation spectrum of the p53 gene was shown to differ significantly between tumour types, indicating that the origins of mutations are distinct in different malignancies. The mutations were analysed by type and position to determine whether any feature of the spectrum could indicate possible factors responsible for the generation of the p53 mutations observed. In this respect the most notable features of the mutation spectrum are observed in hepatocellular, lung, skin and oesophageal cancers.

The mutational spectrum in hepatocellular carcinoma (HCC) contains a high proportion of GC>TA base changes. In three initial studies, 11 of 21 p53 mutations were found to be GC>TA changes in the third nucleotide of codon 249. Since mutations occur at many sites within the p53 gene in the majority of tumour types this positional specificity is exceptional. All of the codon 249 mutations were recorded in two of the studies using tumour material from two areas, Qidong, China and southern Africa (Bressac *et al.*, 1991; Hsu *et al.*, 1991). Both of these areas have a high frequency of HCC thought to be caused in part by the prevalence of hepatitis B virus (HBV) and aflatoxin B1, both known risk factors for the disease. Aflatoxin B1 is a fungal carcinogen found as a contaminant in damp-stored cereals, which forms N^7 deoxyguanosine adducts and induces base substitutions, mostly GC>TA transversions (McMahon *et al.*, 1990). No GC>TA changes at codon 249 were found in the third study (Murakami *et al.*, 1991b) which used tumour material from Japanese patients, who have a low risk of dietary aflatoxin B1 intake.

Further studies strengthened the proposed link between HBV, aflatoxin B1 exposure and p53 mutation by looking specifically for base changes at codon 249 (Hayward *et al.*, 1991; Ozturk *et al.*, 1991; Scorsone *et al.*, 1992). No such mutations were found in HCCs from Australian patients, who have a low risk of exposure to aflatoxin B1, (Hayward *et al.*, 1991) while 21 of 36 (58%) of HCCs from a second series of patients from Qidong contained codon 249 mutations (Scorsone *et al.*, 1992). The presence of the codon 249 mutation was correlated with high risk exposure to HBV and aflatoxin B1 in HCCs from patients in 14 different countries (Ozturk *et al.*, 1991). Comparison of two similar populations of HBV positive patients from neighbouring countries indicated that exposure to aflatoxin B1 was strongly associated with the presence of the codon 249 base change (Ozturk *et al.*, 1991) while HBV infection alone did not seem to correlate with the codon 249 mutation (Hayward *et al.*, 1991; Ozturk *et al.*, 1991).

From these observations, it was proposed that while aflatoxin B1 was strongly associated with the 249 mutation the absence of the 249 mutation in any non-HBV related HCC analysed implicated HBV as a necessary co-factor (Ozturk *et al.*, 1991). The absence of the 249 mutation in aflatoxin B1-induced tumours in non-human primates (Fujimoto *et al.*, 1992a) and in aflatoxin B1-induced hepatic hyperplastic nodules in rats (Hulla *et al.*, 1993) also suggested a role for HBV. However the identification of 249 mutations in HBV negative patients from areas of low risk of aflatoxin B1 exposure questions the role of both risk factors in the generation of this specific change (Oda *et al.*, 1992).

Of 15 p53 mutations identified in squamous cell carcinomas of the skin (SCC), 3 were double CC>TT base changes and the remaining mutations, mainly C>T transitions, occurred at dipyrimidine sequences (Brash *et al.*, 1991). Similarly 7 mutations found in 14 basal cell carcinomas of the skin were all GC>AT transitions (Rady *et al.*, 1992). These types of change are characteristic of UV mutagenesis (Miller 1985) and UV exposure is a well documented disease risk factor for skin cancer. Analysis of p53 mutations in lung and oesophageal cancer, two malignancies associated with tobacco smoking, reveals a high frequency of GC>TA transversions. Tobacco smoke contains benzo(a)pyrene, a carcinogen able to induce GC>TA base changes (Chen *et al.*, 1990b; Andersson 1992). However, although lifetime cigarette consumption has been shown to be associated with non-

small cell lung tumours containing p53 mutations in Japanese patients (Suzuki *et al.*, 1992), no such association was found in U.S. patients (Chiba *et al.*, 1990).

A study of lung tumours from miners exposed to radon, an inert gas which decays to two alpha particle-emitting radioisotopes, revealed a p53 spectrum different to that determined in other lung tumour studies. Despite all the patients being smokers, the most common mutation type seen in lung cancer, GC>TA in the non-coding strand, was not found in any of the tumours examined. The spectrum observed was assumed to be influenced by the exposure to radon with the most frequent base substitution being GC>TA in the coding strand (Vahakangas *et al.*, 1992). Oesophageal tumours have one of the highest frequencies of AT>TA transversions in any solid tumour type and this has been linked to exposure to urethan (Harris 1991), a carcinogen contaminant of alcoholic beverages known to be able to induce AT>TA base changes (Bonham *et al.*, 1989). These examples demonstrate that it is possible for this type of analysis to implicate external carcinogens in the mutation of the p53 gene and thus in the development of solid tumours (Vogelstein and Kinzler 1992).

5.3.3 Determining a p53 mutation spectrum in breast cancer.

In order to determine the types of changes prevalent in sporadic breast cancer 125 tumour and cell line p53 mutations from this and other studies were combined and analysed (Nigro *et al.*, 1989; Bartek *et al.*, 1990a; Prosser *et al.*, 1990; Borresen *et al.*, 1991; Chen *et al.*, 1991a; Devilee P., unpublished data; Davidoff *et al.*, 1991a; Davidoff *et al.*, 1991b; Kovach *et al.*, 1991; Osborne *et al.*, 1991; Varley *et al.*, 1991; Mazars *et al.*, 1992b; Moll *et al.*, 1992; Runnebaum *et al.*, 1991; Sommer *et al.*, 1992; Thompson *et al.*, 1992; Thor *et al.*, 1992) (Table 5.4). Since this study aims to determine factors influencing the types of somatic change occurring within breast tissue, constitutive p53 mutations should not be included. However, the majority of the above studies do not report the screening of the corresponding lymphocyte or normal tissue DNA for the presence of the p53 mutation. Following the identification of 40 mutations in sporadic breast tumours only one was found to be constitutional (Prosser *et al.*, 1990; Coles *et al.*, 1992; Prosser *et al.*, 1992; Thompson *et al.*, 1992). Therefore it could be expected that few of the remaining 85 mutations detected by other

Table 5.4 Codon number and specific alteration for breast tumour mutations

Codon Number	Codon Change	Nucleotide Change	CpG Dinucleotide	Source	Reference
128	CCT > CCG	T > G		Tumour	Borresen *et al.*, 1991
128	CCT > TCT	C > T	non	Tumour	Borresen *et al.*, 1991
132	AAG > CAG	A > C		Cell line	Bartek *et al.*, 1990
132	AAG > AAT	G > T		Tumour	Mazars *et al.*, 1992b
134	TTT > CTT	T > C		Tumour	Borresen *et al.*, 1991
134	TTT >TTA	T > A		Tumour	Thor *et al.*, 1992
135	TGC > TGG	C > G		Tumour	Mazars *et al.*, 1992b
136	CAA > GAA	C > G		Tumour	Prosser *et al.*, 1991
138	GCC > GTC	C > T	non	Tumour	Moll *et al.*, 1992
138	GCC > GTC	C > T	non	Tumour	Moll *et al.*, 1992
139	AAG > AAT	G > T		Tumour	Mazars *et al.*, 1992b
141	TGC > TAC	G > A	non	Tumour	This study
149	CCC > TCC	C > T	non	Tumour	Thor *et al.*, 1992
151	CCC > TCC	C > T	non	Tumour	Chen *et al.*, 1991a
152	CCG > TCG	C > T	non	Tumour	Prosser *et al.*, 1991
157	GTC > TTC	G > T		Tumour	Prosser *et al.*, 1991
157	GTC > TTC	G > T		Cell line	Kovach *et al.*, 1991
157	GTC > TTC	G > T		Tumour	Mazars *et al.*, 1992b
163	TAC > AAC	T > A		Tumour	This study
163	TAC > TGC	A > G		Tumour	Davidoff *et al.*, 1991a
163	TAC > AAC	T > A		Tumour	Sommer *et al.*, 1992
164	AAG > CAG	A > C		Tumour	Prosser *et al.*, 1991
164	AAG > GAG	A > G		Tumour	Thor *et al.*, 1992
165	CAG > CTG	A > T		Tumour	Mazars *et al.*, 1992b
175	CGC > CAC	G > A	CpG	Tumour	This study
175	CGC > CAC	G > A	CpG	Tumour	This study
175	CGC > CAC	G > A	CpG	Tumour	Varley *et al.*, 1991
175	CGC > CAC	G > A	CpG	Tumour	Borresen *et al.*, 1991
175	CGC > CTC	G > T		Tumour	Prosser *et al.*, 1991
175	CGC > CAC	G > A	CpG	Cell line	Kovach *et al.*, 1991
175	CGC > CAC	G > A	CpG	Tumour	Sommer *et al.*, 1992
175	CGC > CAC	G > A	CpG	Tumour	Thor *et al.*, 1992
176	TGC > TTC	G > T		Tumour	Mazars *et al.*, 1992b
179	CAT > GAT	C > G		Tumour	Prosser *et al.*, 1991
179	CAT > TAT	C > T	non	Tumour	This study
179	CAT > GAT	C > G		Tumour	Kovach *et al.*, 1991
182	TGC > TGA	C > A		Tumour	This study
186	GAT > TAT	G > T		Tumour	This study
187	GGT > TGT	G > T		Tumour	Prosser *et al.*, 1991
192	CAG > TAG	C > T	non	Tumour	This study
193	CAT > CCT	A > C		Tumour	Devilee, P.*
194	CTT > CGT	T > G		Tumour	This study
194	CTT > CGT	T > G		Tumour	This study
194	CTT > CGT	T > G		Tumour	This study
194	CTT > TTT	C > T	non	Cell line	Nigro *et al.*, 1989
196	CGA > CCA	G > C		Tumour	Devilee, P.*
196	CGA > TGA	C > T	CpG	Tumour	Mazars *et al.*, 1992b

Table 5.4 (continued)

Codon Number	Codon Change	Nucleotide Change	CpG Dinucleotide	Source	Reference
208	GAC > GTC	A > T		Tumour	This study
213	CGA > TGA	C > T	CpG	Tumour	This study
213	CGA > TGA	C > T	CpG	Tumour	This study
213	CGA > TGA	C > T	CpG	Tumour	Sommer *et al.*, 1992
232	ATC > AGC	T > G		Tumour	Mazars *et al.*, 1992b
234	TAC > TAA	C > A		Cell line	Runnebaum *et al.*, 1991
238	TGT > TTT	G > T		Tumour	Osborne *et al.*, 1991
238	TGT > TTT	G > T		Tumour	Borresen *et al.*, 1991
238	TGT > TCT	G > C		Tumour	Thor *et al.*, 1992
237	ATG > ATT	G > T		Tumour	This study
237	ATG > AAG	T > A		Tumour	Borresen *et al.*, 1991
237	ATG > ATA	G > A	non	Tumour	Davidoff *et al.*, 1991a
239	AAC > GAC	A > G		Tumour	This study
241	TCC > GCC	T > G		Tumour	Mazars *et al.*, 1992b
242	TGC > TTC	G > T		Tumour	Thompson *et al.*, 1992
245	GGC > GAC	G > A	non	Tumour	Davidoff *et al.*, 1991a
245	GGC > GTC	G > T		Tumour	Borresen *et al.*, 1991
245	GGC > GAC	G > A	non	Tumour	This study
245	GGC > TGC	G > T		Tumour	Moll *et al.*, 1992
248	CGG > TGG	C > T	CpG	Tumour	Borresen *et al.*, 1991
248	CGG > CAG	G > A	CpG	Tumour	Davidoff *et al.*, 1991a
248	CGG > CAG	G > A	CpG	Tumour	Davidoff *et al.*, 1991b
248	CGG > CAG	G > A	CpG	Tumour	Sommer *et al.*, 1992
248	CGG > TGG	C > T	CpG	Tumour	Mazars *et al.*, 1992b
248	CGG > TGG	C > T	CpG	Tumour	Mazars *et al.*, 1992b
249	AGG > AGC	G > C		Cell line	Bartek *et al.*, 1990
249	AGG > AGT	G > T		Tumour	Moll *et al.*, 1992
249	AGG > AGC	G > C		Tumour	Mazars *et al.*, 1992b
250	CCC > GCC	C > G		Tumour	Thompson *et al.*, 1992
254	ATC > AAC	T > A		Tumour	Davidoff *et al.*, 1991b
255	ATC > AAC	T > A		Tumour	Mazars *et al.*, 1992b
265	CTG > CCG	T > C		Tumour	Thompson *et al.*, 1992
266	GGA > GTA	G > T		Tumour	Davidoff *et al.*, 1991b
272	GTG > ATG	G > A	non	Tumour	Mazars *et al.*, 1992b
272	GTG > ATG	G > A	non	Tumour	Mazars *et al.*, 1992b
273	CGT > CAT	G > A	CpG	Cell line	Nigro *et al.*, 1989
273	CGT > CTT	G > T		Tumour	This study
273	CGT > CAT	G > A	CpG	Tumour	This study
273	CGT > CAT	G > A	CpG	Tumour	Borresen *et al.*, 1991
273	CGT > CAT	G > A	CpG	Tumour	Sommer *et al.*, 1992
273	CGT > CCT	G > C		Tumour	Sommer *et al.*, 1992
275	TGT > TGG	T > G		Tumour	Sommer *et al.*, 1992
276	GCC > CCC	G > C		Tumour	This study
278	CCT > GCT	G > C		Tumour	Davidoff *et al.*, 1991a
280	AGA > ACA	G > C		Tumour	Osborne *et al.*, 1991
280	AGA > ACA	G > C		Tumour	Kovach *et al.*, 1991
280	AGA > GGA	A > G		Tumour	Thompson *et al.*, 1992

Table 5.4 (continued)

Codon Number	Codon Change	Nucleotide Change	CpG Dinucleotide	Source	Reference
280	AGA > AAA	G > A	non	Cell line	Bartek *et al.*, 1990
280	AGA > ACA	G > C		Tumour	Sommer *et al.*, 1992
281	GAC > GGC	A > G		Tumour	Borresen *et al.*, 1991
281	GAC > GGC	A > G		Tumour	Thompson *et al.*, 1992
282	CGG > CTG	G > T		Tumour	This study
282	CGG > CCG	G > C		Tumour	Varley *et al.*, 1991
282	CGG > CTG	G > T		Tumour	Davidoff *et al.*, 1991a
283	CGC > CCC	G > C		Tumour	Thompson *et al.*, 1992
285	GAG > AAG	G > A	non	Tumour	This study
285	GAG > AAG	G > A	non	Tumour	Osborne *et al.*, 1991
285	GAG > AAG	G > A	non	Cell line	Bartek *et al.*, 1990
285	GAG > AGG	G > A	non	Tumour	Moll *et al.*, 1992
306	CGA > TGA	C > T	CpG	Tumour	Mazars *et al.*, 1992b
307	GCA > ACA	G > A	non	Tumour	Thompson *et al.*, 1992
342	CGA > TGA	C > T	CpG	Tumour	Moll *et al.*, 1992
intron 9	Splice mutation	G > A	non	Tumour	Sommer *et al.*, 1992

Codon	Codon Change	Deletion	Source	Reference
140	GAC > GΔC	AT base pair	Tumour	This study
144	AGC codon deletion	3 base pairs	Tumour	Sommer *et al.*, 1992
167	CAG > CAΔ	GC base pair	Tumour	Varley *et al.*, 1991
172	GTT > GT	TA base pair	Tumour	Borresen *et al.*, 1991
175 -180	Deletion 6 codons	18 base pairs	Tumour	Davidoff *et al.*, 1991a
201	TTG > TTΔ	GC base pair	Tumour	Prosser *et al.*, 1991
233 - 235	Frameshift deletion	8 base pairs	Tumour	Sommer *et al.*, 1992
235 - 239	Frameshift deletion	14 base pairs	Tumour	Kovach *et al.*, 1991
241	TCC codon deletion	3 base pairs	Tumour	Moll *et al.*, 1992
252 - 254	Deletion 3 codons	9 base pairs	Tumour	Sommer *et al.*, 1992
255	ATC codon deletion	3 base pairs	Tumour	This study
265 - int8	Frameshift deletion	11 base pairs	Tumour	Mazars *et al.*, 1992b
279 - 287	Frameshift deletion	23 base pairs	Tumour	Mazars *et al.*, 1992b
329	ACC > ACΔ	CG base pair	Tumour	Kovach *et al.*, 1991
exon 11	Deletion 30 bp	30 base pairs	Tumour	Osborne *et al.*, 1991

CpG - GC>AT transition at a CpG dinucleotide site
non - GC>AT transition at any non-CpG dinucleotide
* - unpublished data
Δ - single base deletion

studies would be constitutive.

5.3.4 Analysis of base changes in the breast cancer p53 mutation spectrum.

As seen in a wide range of malignancies, the most frequent type of mutation occurring in the p53 gene is a single base pair substitution (Hollstein *et al.*, 1991a; Caron de Fromentel and Soussi 1992). This is observed in breast cancers alone where 110/125 (88%) of mutations are single base pair substitutions and the remaining 15/125 (12%) are deletions (Table 5.4). Substitutions resulting in a missense mutation, producing a protein differing in a single amino acid, account for 81% (101/125) of changes. Those resulting in a stop codon, producing a truncated mRNA and p53 protein, account for 7% (9/125).

The p53 base substitutions identified in breast tumours have been grouped into mutation types and compared with those from other solid tumours (Tables 5.5 and 5.6). The mutations identified from other solid tumours originate from carcinomas of the colon (Baker *et al.*, 1989; Nigro *et al.*, 1989; Baker *et al.*, 1990a; Rodrigues *et al.*, 1990; Skirasawa *et al.*, 1991; Cunningham *et al.*, 1992), lung (N-SCLC) (Nigro *et al.*, 1989; Takahashi *et al.*, 1989; Chiba *et al.*, 1990; Iggo *et al.*, 1990; Lehman *et al.*, 1991; Bodner *et al.*, 1992; Kishimoto *et al.*, 1992; Miller *et al.*, 1992; Mitsudoni *et al.*, 1992), lung (SCLC) (Nigro *et al.*, 1989; Takahashi *et al.*, 1989; Hensel *et al.*, 1991; Lehman *et al.*, 1991; Bodner *et al.*, 1992; D'Amico *et al.*, 1992; Miller *et al.*, 1992; Sameshima *et al.*, 1992b), oesophagus (Hollstein *et al.*, 1990; Bennet *et al.*, 1991; Carson *et al.*, 1991; Hollstein *et al.*, 1991a), liver (Bressac *et al.*, 1991; Hsu *et al.*, 1991; Murakami *et al.*, 1991; Challen *et al.*, 1992; Oda *et al.*, 1992; Sheu *et al.*, 1992), ovary (Marks *et al.*, 1991; Mazars *et al.*, 1991; Okamoto *et al.*, 1991a; Yoginuma and Westphal 1992), brain (Nigro *et al.*, 1989; Mashiyama *et al.*, 1991; Frankel *et al.*, 1992; Fults *et al.*, 1992; Sidransky *et al.*, 1992b; von Deimling *et al.*, 1992) and bladder (Hollstein *et al.*, 1991a; Sidransky *et al.*, 1991; Fujimoto *et al.*, 1992b). Constitutional p53 mutations occurring were also analysed (Malkin *et al.*, 1990; Srivastava *et al.*, 1990; Law *et al.*, 1991; Metzger *et al.*, 1991; Santibanez-Koref *et al.*, 1991; Borresen *et al.*, 1992; Felix *et al.*, 1992a; Iavarone *et al.*, 1992; Kovar *et al.*, 1992; Malkin *et al.*, 1992; Prosser *et al.*, 1992; Sameshima *et al.*, 1992a; Sidransky *et al.*, 1992a; Toguchida *et al.*, 1992; Malkin and Friend 1993).

Table 5.5 Frequency of specific types of p53 mutation in breast tumours, constitutive cancer syndromes and other solid tumours.

	GC>CG	GC>TA	GC>AT		AT>GC	AT>CG	AT>TA	Total
				CpG Non				
Breast p53 mutations identified in this study	1	5	10	6 4	1	3	2	22
Breast p53 mutations identified from Scottish patients	5	9	12	6 6	4	4	2	36
Total breast p53 mutations	17	22	45	23 22	8	10	8	110
Colon p53 mutations	2	0	36	30 6	8	0	2	48
Lung p53 mutations (N-SCLC)	19	49	37	12 25	8	5	8	126
Lung p53 mutations (SCLC)	6	25	11	6 5	4	5	4	55
Brain p53 mutations	2	4	30	20 10	6	1	0	43
Oesophagus p53 mutations	0	9	16	7 9	4	3	5	37
Bladder p53 mutations	5	2	8	6 2	3	3	0	21
Liver p53 mutations	4	30	13	6 7	14	4	14	79
Ovary p53 mutations	4	3	8	2 6	1	2	1	19
Total p53 mutations in solid tumours	59	144	204	113 91	56	33	42	538
Constitutive mutations (mainly Li-Fraumeni)	1	2	20	17 3	2	2	1	28

Table 5.6 Frequency of specific types of base substitution in breast tumours, constitutive cancer syndromes and other solid tumours expressed as a percentage.

	GC>CG	GC>TA	GC>AT		AT>GC	AT>CG	AT>TA	Total
			CpG	Non				
Breast p53 mutations identified in this study	5	23	28	18	5	17	9	100
Breast p53 mutations identified from Scottish patients	14	25	17	17	11	11	5	100
Total breast p53 mutations	16	20	21	20	7	9	7	100
Colon p53 mutations	4	0	63	12	17	0	4	100
Lung p53 mutations (N-SCLC)	15	39	10	20	6	4	6	100
Lung p53 mutations (SCLC)	11	46	11	9	7	9	7	100
Brain p53 mutations	5	9	47	23	14	2	0	100
Oesophagus p53 mutations	0	24	19	24	11	8	14	100
Bladder p53 mutations	24	10	28	10	14	14	0	100
Liver p53 mutations	5	38	7	9	18	5	18	100
Ovary p53 mutations	21	16	10	32	5	11	5	100
Total p53 mutations in solid tumours	11	27	21	17	10	6	8	100
Constitutive mutations (mainly Li-Fraumeni)	4	7	60	11	7	7	4	100

One of the most notable features of the p53 mutational spectra is the preponderance of base substitutions at GC base pairs. If all alterations occurred randomly then the frequency of the changes seen at GC base pairs would be expected to reflect the GC content of the gene. While the overall content of GC in the p53 gene sequence is 56% (Chumakov P.M. EMBL access number X54156 for HSP53G), 76% of the base substitutions in all solid tumours occur at GC base pairs. A similar figure of 77% is observed when analysing breast tumours alone (table 5.6). The most frequent types of base pair substitution observed in breast tumours all occur at GC base pairs: GC>AT transitions (41%) and GC>TA (20%) and GC>CG (16%) transversions.

GC>AT transitions in breast cancer occurs at approximately equal frequency at both CpG dinucleotides (21%) and at cytosine and guanine residues not contained in this sequence conformation (20%). 5-methylcytosine is present in the genome at a frequency of 1% and occurs mainly at CpG dinucleotide sequences. GC>AT transitions at CpG dinucleotides are thought to arise by the deamination of 5-methylcytosine to thymine (Bird et al., 1980). Although it is not known which CpG residues are methylated in breast tissue, the CpG dinucleotides at codons 170, 175, 181, 185/6 and 273 have been shown to be methylated in white blood cell, sperm, liver, muscle, urothelium and cultured skin fibroblast DNA (Rideout *et al.*, 1990). In accordance with this, 7/8 mutations at codon 175 and 4/6 mutations at codon 273 are consistent with the deamination of 5-methylcytosine to thymine at a CpG dinucleotide. Although 21% of the base substitutions in breast cancer could be due to this endogenous mechanism it is clearly not as important a factor as it appears to be in colon (63%) and brain (47%) tumours or constitutional cancer syndromes (60%).

The highest frequencies of GC>TA transversions are seen in oesophageal (24%), liver (38%) and lung (41%) cancers and a similar preponderance is found in breast cancer (22/108, 20%). As previously noted, external carcinogens are thought to play an important part in the generation of these tumours. The nucleotide G is known to be a preferential target for most chemical carcinogen-induced mutations (Kriek *et al.*, 1984). Therefore the prevalence of GC>TA base changes implies that external carcinogens have a role in the development of breast tumours.

Of 84 base substitutions at GC base pairs 59 occur with the guanine residue in the non-coding strand. This strand bias is seen in GC>CG, GC>TA and GC>AT mutation types although it

is most pronounced for GC>TA transversions (table 5.7). It is possible that 59/125 (47%) of all mutations in breast tumours are point mutations occurring at the guanine residues in the non-coding strand. However 14/27 GC>AT transitions occurring at CpG dinucleotides could more realistically be viewed as alterations caused by the deamination of 5 methylcytosine to thymine in the coding strand. Nevertheless the remaining 45/125 (36%) mutations occurring at guanine in the non-coding strand is in excess of expectation.

Table 5.7 Strand bias in GC base pair substitutions.

	GC > CG	GC > TA	GC > AT
G in the non coding strand	12	20	27
C in the non-coding strand	5	2	18
Total	17	22	45

Mutagenesis experiments in mammalian cells have shown that the non-transcribed strand is more frequently the site of damage and this is thought to be due to either the preferential repair of the coding over the non-coding strand (Mellon *et al.*, 1987) or increased susceptibility of the non-coding strand to damage. This bias is also seen in lung tumours of both the small cell and non-small cell types (Hollstein *et al.*, 1991a). All of the N-SCLC and 23/25 SCLC GC>TA mutations involved nucleotide base pairs containing guanine in the non-coding strand. This strand bias was also observed with GC>CG transversions in N-SCLC and SCLC and with GC>AT transitions in SCLC. The number of changes seen at AT base pairs is too small to indicate any strand bias in this type of mutation (table 5.5).

5.3.5 Analysis of position in the breast cancer p53 mutation spectrum.

The preponderance of a particular type of base substitution in a spectrum can implicate many potential origins, eg GC>TA transversions can arise thought DNA polymerase infidelity (Loeb and Cheng 1990), 8-hydroxyguanine adduct formation by oxy-radical damage (Wood *et al.*, 1990) and N^2 -BPDE deoxyguanine adduct formation by benzo(a)pyrene (Chen et al., 1990b). Therefore it

is the positional specificity of mutations that will provide the most information on specific factors involved in p53 mutagenesis (Hollstein *et al.*, 1991a).

The 125 breast cancer p53 mutations occur between exons 5 and 11: 43/125 (34%) in exon 5; 14/125 (11%) in exon 6; 32/125 (25%) in exon 7; 31/125 (25%) in exon 8; 2/125 (2%) in exon 9; 1/125 (1%) in intron 9; 1/125 (1%) in exon 10; and 1/125 (1%) in exon 11. The missense mutations are found in 54 different codons and the deletions involve a further 69 codons. Four regions of the p53 gene showing high frequency of mutation, known as hotspot regions (HSRs), have been determined (Nigro *et al.*, 1989). These four HSRs (A to D) show good correlation with four of the five highly conserved domains (HCDs II-V) found in the gene (Soussi *et al.*, 1990). Seventy five of 110 (68%) point substitutions and 5/9 (56%) deletions are found within these four HCDs. Of the remaining missense mutations, 20/34 are found at codons conserved from Xenopus to mammals and 13/34 are found at codons conserved between mammals. A fifth HSR, HSR A', has been determined between the codons 151 and 159 (Caron de Fromentel and Soussi 1992). This HSR, however, is the location of only 5/125 (4%) breast cancer mutations.

Hotspot codons are particularly interesting as they can indicate the influence of a potent factor on the mutation spectrum of the p53 gene. The determination of a hotspot at codon 249 strongly suggested a role for aflatoxin B1 in the development of HCC. Over 50% of mutations occur at codons 175, 248, 273 and 282 in colon tumours. These codons contain CpG dinucleotide sites and all of the changes at these sites are GC>AT transitions, implicating 5-methylcytosine as an endogenous mutagen involved in the generation of a high number of colon p53 mutations.

The most frequent sites for breast cancer mutations are codons 175 (8), 194 (4), 245 (4), 248 (6), 273 (6), 280 (5) and 285 (4). Together they account for only 37/125 (30%) of the mutations identified. Codons 175, 245, 248 and 273 are found to be mutated in a wide variety of malignancies including cancers of the colon, brain, oesophagus, liver and cervix (Hollstein *et al.*, 1991a; Caron de Fromentel and Soussi 1992). Mutations at codon 194 have only been identified in two oesophageal carcinomas (Hollstein *et al.*, 1991b), one small cell lung tumour (Takahashi *et al.*, 1989) and a B-cell chronic lymphocytic leukemia (B-CLL) (Gaidano *et al.*, 1991). Codon 280 has been shown to be mutated in an oesophageal tumour (Hollstein *et al.*, 1991b), a non-small cell lung tumour (Kishimoto

et al., 1992), a rhabdomyosarcoma cell line (Felix *et al.*, 1991b) and a pancreatic carcinoma cell line (Barton *et al.*, 1991). Mutations at codon 285 have been detected in four non-small cell lung tumours (Bodner *et al.*, 1992; Mitsudoni *et al.*, 1992). Identification of three codons mutated in breast (13/110, 12%), oesophageal (3/37, 8%) and non-small cell lung tumours (5/126, 4%), but rarely in other malignancies, may indicate a common mechanism of mutation altering the p53 gene in these types of tissue. Although this kind of analysis is limited due to the number of mutations identified in certain tumour types the characterisation of further p53 mutations will aid in the identification of common sites of mutation in different malignancies.

5.3.6 Discussion

Analysis of the p53 mutation spectrum in breast cancer reveals a high frequency of mutations at G residues, suggesting the involvement of external carcinogens. However it must be remembered that the p53 spectrum may not simply be the product of the interaction between exogenous factors and DNA. The differences observed between mutation spectra of different tumour types could be due largely to endogenous factors. Recent studies have proposed that exogenous factors, in the doses normally encountered, would have little or no discernible effect when added to the huge carcinogenic load originating from endogenous factors (Rudiger 1990). Thus differences between p53 mutation spectra could be accounted for by variation in endogenous factors such as 5-methylcytosine content (Ehrlich *et al.*, 1982), type and efficiency of DNA repair mechanisms (Harris 1989) and concentration of various endogenous mutagenic compounds such as reducing sugars (Lee and Cerami 1990), lipids and lipid precursors (Esterbauer *et al.*, 1990) and amino acid derivatives (Glatt 1990) between tissue types.

A second consideration is that the p53 spectra could simply be an effect of tissue specific mutation selection. Mutant p53 proteins have differing properties (Hinds *et al.*, 1990; Halvey *et al.*, 1990) and it has been hypothesised that the growth advantage conferred by certain p53 proteins is dependent on the cell type in which the mutation occurs (Levine *et al.*, 1991). Mutations not appearing in the spectrum of a particular tumour type may be lethal or may not offer any selective advantage to that cell type. Thus the hotspot codons, 175, 248, 273 and 282, which are frequently

mutated in colon cancer, could reflect strong cell-specific selection, rather than the deamination of 5-methylcytosine at these sites. The codon 249 mutation occurring in HCCs could also be hepatocyte specific rather than mutagen specific (Vogelstein and Kinzler 1992).

A possible example of a non-selectable mutation is demonstrated by GC>AT substitutions occurring at the CpG dinucleotide at codon 175 (Caron de Fromentel and Soussi 1992). Twenty two transitions found at this site in a range of cancers exclusively involve the G residue of the dinucleotide, resulting in an amino acid change of arginine to histidine. Transitions involving the C residue would produce an arginine to cysteine substitution. The CpG dinucleotides at codons 248 and 273, which are mutated at a high frequency in human tumours, are mutated at the C and G residues at equal frequencies suggesting that the codon 175 Arg to Cys mutation does not confer any growth advantage.

The constitutional p53 mutations found so far in familial cancer syndromes such as LFS could be the result of a specific type of selection. These mutations show unusual clustering in exon seven and 68% (23/34) are located between the codons 241 and 286. The mutation spectrum reveals two hotspots, one at codon 245 (4/34, 12%) and another at codon 248 (7/34, 21%) and 57% (16/28) of base substitutions occur at CpG dinucleotides. The mutant p53 proteins have been shown to have distinct properties, such as differences in growth suppression (Frebourg *et al.*, 1992), protein expression (Srivastava *et al.*, 1992) and ability to drive the wild type p53 protein into the mutant conformation (Milner and Metcalf 1991), when compared to somatically acquired p53 mutants. These distinct characteristics suggest that only certain p53 mutations will be tolerated when present constitutively. The spectrum of constitutional p53 mutations might therefore reflect selection for these characteristics rather than the influence of exogenous and endogenous mechanisms of mutation.

The analysis of p53 mutations in solid tumours can only implicate external carcinogens if these have a direct effect on p53 mutation. Less than half of the chemicals shown to be carcinogenic in rats actually show mutagenic properties in Salmonella (Ames and Gold 1990). The remaining chemicals are thought to act as mitogens or cytotoxins (Hoel *et al.*, 1988), and influence DNA mutation by an increase in cell division. This increase has been postulated to enhance mutation

formation by endogenous factors in several ways: by allowing adducts to be converted into mutations; by increasing the incidence of mitotic recombination, gene conversion and non-disjunction events, which are important factors in tumour suppressor gene inactivation; and by allowing gene duplication, which can lead to the increased expression of oncogenes. An increase in DNA replication is also accompanied by an increase in the level of ssDNA, which is more susceptible to damage than dsDNA (Ames and Gold 1990). Thus it is possible that a carcinogen acting on breast epithelial cells, because it increases the frequency of mutations caused by endogenous factors and does not effect the DNA directly, will not induce carcinogen-specific mutations.

Identification of specific exogenous and endogenous factors through p53 mutation spectrum analysis relies on the presumption that the mutations arising in the tumour samples have similar origins. The p53 mutations used to determine the breast cancer p53 mutation spectrum originated from patients in Scotland, England, France, the Netherlands, Norway and the USA. The origins of p53 mutations in different patient populations might be expected to reflect exposure to differing carcinogens and variation in internal endogenous factors. Quantitative differences in DNA repair, DNA adduct formation and carcinogen metabolism between individuals have been described and may modify the pattern of p53 mutation (Harris 1989). An example of this might be seen in the analysis of breast cancer where the mutation CTT>CGT at codon 194 accounts for 3/110 (3%) single base substitutions, all of which are found in Scottish patients (3/36, 8%) ($0.1 > P > 0.05$, Fisher's exact test). A higher frequency of p53 deletions, 5/14 (36%) compared to 10/111 (9%) in all other breast tumour studies, has been detected in breast tumours from patients in Rochester, USA (Sommer *et al.*, 1992) ($0.05 > P > 0.01$, Fisher's exact test). Similarly a prominent mutation hotspot at codon 273 found in SCLC samples from American patients was not detected in SCLC samples from Japanese patients (Chiba *et al.*, 1990; Mitsodomi *et al.*, 1992; Suzuki *et al.*, 1992). Thus specific factors inducing p53 mutations in one population may not be present in another and therefore might not be apparent in an analysis using p53 mutations occurring in differing populations.

For the spectra of p53 mutations to be of any use in determining specific causal factors, the type and positions of p53 mutations induced by specific carcinogens need to be determined. While

most of this kind of work has been done on the ras oncogene in rodents, similar studies in humans would need to rely upon *in vitro* treatment of human cells with chemical carcinogens. Studies similar to those investigating the mutation spectra of the hypoxanthine-guanine phosphoribosyl transferase (HPRT), adenine phosphoribosyl transferase (APRT) and thymidine kinase (TK) genes would be ideal although, unlike these genes, there is no convenient selection test for mutated p53 genes (Clive *et al.*, 1972; Albertini and DeMars 1973; Jones and Sargent 1974). Experimental systems in which immortalised cells arising after chemical treatment are tested for p53 mutation on the basis of immunohistochemistry (Maehle *et al.*, 1992) or transcriptional activation (Frebourg *et al.*, 1992) could be utilised. However, p53 mutations occurring *in vitro* would perhaps not reflect those occurring *in vivo* (Harvey and Levine 1991; Rittling and Denhardt 1992). The feasibility and productivity of such an approach must be considered before further studies in this direction are undertaken. Characterisation of the p53 mutations induced by a single chemical would be a major task, even when using non-sequencing methods such as the SSCP and HOT techniques. Considering that more than 50% of all chemicals tested are carcinogenic (Ames and Gold 1990) the effort needed for the characterisation of even a small number would be enormous.

Given that the p53 spectrum in breast cancer, as it stands, shows no features as notable as the hotspots in HCC and colon cancer, identification of exogenous or endogenous factors by this method may not be particularly efficient. In addition, those exogenous factors implicated in the carcinogenic process by p53 mutation analysis (benzo(a)pyrene in lung cancer, aflatoxin B1 exposure in HCC, urethan in oesophageal cancer and UV exposure in skin cancers) had previously been identified as risk factors by epidemiological approaches (Selikoff *et al.*, 1968; Yeh *et al.*, 1989; Yu *et al.*, 1980) and mutagenesis studies (Miller 1985, McMahon *et al.*, 1990; Chen *et al.*, 1990b). This suggests that any exogenous factor potent enough to affect the mutation spectrum of the p53 gene so as to allow it's identification might already have been implicated by previous epidemiology and mutagenesis studies.

Chapter 6

Correlation of p53 mutation and LOH with pathological/clinical parameters

6.1 Prognostic makers in breast cancer.

To date the most informative factors for predicting disease recurrence and overall survival in breast cancer patients are the metastatic spread of the tumour to axillary lymph nodes (Shek and Godolphin 1988) and tumour stage (TNM), which combines information on spread to axillary lymph nodes with tumour size and occurrence of distant metastases (Donegan 1992). Disease recurrence in the 10 years following primary treatment occurs in 20-30% of patients without histologically involved axillary nodes in comparison to 75% of patients with histologically involved axillary nodes (Fisher 1984). Oestrogen (ER) and progesterone receptor (PR) levels, tumour grade and histological type can also be used as indicators of prognosis (Knight *et al.*, 1977; Gallager 1984; Parl *et al.*, 1984; Le Doussal *et al.*, 1989). In addition ER and PR levels can be used to identify tumours with a high probability of response to endocrine or hormone therapy. A favourable response to such treatment is found in three quarters of patients with ER and PR positive tumours and in 14% of patients with ER and PR negative tumours (McGuire *et al.*, 1978).

The search for a marker capable of predicting disease recurrence and overall survival independently of lymph node involvement has been the aim of many studies. As metastatic breast cancer is incurable, this is particularly important in axillary node negative (ANN) breast cancer where such a marker could be used to identify those patients at high risk of recurrence (McGuire *et al.*, 1992). Adjuvant endocrine therapy and chemotherapy have been shown to increase disease free survival significantly in node negative patients (Mansour *et al.*, 1989; Fisher *et al.*, 1989; Nayfield *et al.*, 1991). A marker capable of predicting the risk of recurrence could be used to separate patients into high and low risk groups. High risk patients could benefit from adjuvant therapy while low risk patients could avoid unnecessary and expensive treatment along with potential side effects.

Since tumorigenesis is a process resulting from an accumulation of genetic alterations affecting cellular growth control, tumour behaviour might therefore be expected to reflect these underlying genetic changes. Determination of these changes and their association with pathological and clinical characteristics could indicate the origins of certain tumour characteristics. Ultimately these associations may be able to divide tumours into subgroups whereby their clinical behaviour can

be reliably predicted and the appropriate treatment more successfully applied (McGuire *et al.*, 1991; Wolman *et al.*, 1992).

Many studies have attempted to associate tumour and patient characteristics with alterations of specific oncogenes or LOH at specific sites of the genome in the hope of identifying a reliable clinical marker (Callahan 1989). The over-expression of c-myc has been correlated with oestrogen receptor level, (Mizukami *et al.*, 1992), patient age (Escot *et al.*, 1986; Spandidos *et al.*, 1989), progesterone receptor level (Adane *et al.*, 1989), metastatic spread to lymph nodes (Guerin *et al.*, 1988), and poor prognosis (Varley *et al.*, 1987). Over-expression of the ras oncogene has been correlated with oestrogen receptor level (Muzukami *et al.*, 1992), histological grade (Agnantis *et al.*, 1986a) and tumour invasion (Horan Hand *et al.*, 1984). Alteration of the int-2 gene has been correlated with oestrogen receptor level (Fantl *et al.*, 1990; Borg *et al.*, 1991; Berns *et al.*, 1992), progesterone receptor level (Adane *et al.*, 1989) and metastatic spread to lymph nodes (Adane *et al.*, 1989). The oncogene on which most work has been done is the neu/c-erbB2/HER oncogene, the amplification/overexpression of which has shown correlation with poor prognosis in node positive and, in some studies, node negative tumours (Gullick 1990; Perren 1991; Borg 1992).

The following parameters have been shown to be significantly associated with LOH on specific chromosomes: tumour size with LOH on 13 and 11p (Mackay *et al.*, 1988b; Andersen *et al.*, 1992); metastatic spread to lymph nodes with LOH on 1q, 11p and 6q (Ali *et al.*, 1987; Devilee *et al.*, 1991a; Chen *et al.*, 1989); histological tumour type with LOH on 13q (Devilee *et al.*, 1991a); menopausal status with LOH on 17q (Borresen *et al.*, 1991); age at disease onset with LOH on 17q (Devilee *et al.*, 1991a); oestrogen receptor level with LOH on 11p, 13q, 17q and 18q (Ali *et al.*, 1987; Mackay *et al.*, 1988b; Cropp *et al.*, 1990; Devilee *et al.*, 1991a); high grade tumours with LOH on 3p, 11 and 18q (Ali *et al.*, 1987; Ali *et al.*, 1989b; Cropp *et al.*,1990); and disease free interval and overall survival with LOH on 7q (Bieche *et al.*, 1992).

The associations determined by the oncogene/LOH studies are not consistently found in every investigation. This variability has been attributed to a number of factors: sampling errors due to small numbers of tumours; different compositions of the patient and tumour populations; varying fractions of normal cell contamination within tumour samples, which is especially relevant in LOH

and mRNA studies; limited periods of post-surgical follow-up; differences in experimental techniques and data analysis; and the failure to consider the effects of post-surgical treatment. Consequently, studies of the above type have been criticised for their approach and guidelines for the setting up of future studies have been recommended (McGuire *et al.*, 1991). Under these guidelines the present attempt to correlate p53 mutation and LOH on chromosome 17p with pathological/clinical parameters, considering the small sample size and limited patient and tumour information, would qualify as 'pilot study'. The results could only be used as an initial indication of the potential of these genetic alterations as a useful prognostic markers.

6.2 Correlation of LOH on chromosome 17 and p53 mutation with pathological/clinical parameters.

In the present study six clinical/pathological parameters were examined for correlation with either LOH on chromosome 17 or p53 mutation. The pathological/clinical parameters were tumour size, metastatic spread to lymph nodes, age of patient at presentation, patient menopausal status, family history of breast disease and tumour oestrogen receptor protein level. Seventy eight tumour samples analysed for mutations in the p53 gene have information on three or more pathological/clinical parameters (Table 6.1). Thirty five tumour samples analysed for LOH using markers near the p53 gene have information on three or more pathological/clinical parameters (Table 6.2) The relationships between the molecular alterations and pathological/clinical parameters were analysed statistically by the χ^2 or Fishers exact (2x2) test using C-stat, an IBM computer statistics package. Tables 6.3 and 6.4 show the probability levels (P value) for the association of LOH and p53 mutation with the pathological/clinical parameters. P values of less than 0.05 were considered statistically significant.

p53 mutation was found to be associated with two pathological/clinical parameters, menopausal status and low levels of oestrogen receptor protein (Table 6.3). No pathological/clinical parameter was associated with LOH at the p53 locus (Table 6.4). This is perhaps not unexpected considering that tumour heterogeneity and normal cell contamination would mask the actual LOH status of the breast tumour samples analysed (sections 3.2 and 3.3).

Table 6.1 Pathological, clinical and molecular data on the breast cancer patients and tumours.

p53 gene	Tumour Number	Size (cm)	Histological Tumour Type	Nodal Status	Patient Age	M/P Status	Family History	ER level (pg)
wt	4	-	-	-	79	Post	No	220
wt	6	3.2	Ductal/NST	10/23	62	-	No	789
wt	7	2.8	Ductal/NST	0/7	34	Pre	No	2
wt	9	9	Ductal/NST	4/5	73	Post	No	-
wt	14	1.4	Ductal/NST	1/17	44	Peri	Yes	51
wt	15	3.0	Ductal/NST	1/17	44	Peri	Yes	39
wt	16	2.0 (B)	Ductal/NST	-	60	-	Yes	80
wt	20	1	Ductal/NST	0/3	72	Post	No	-
wt	22	1.3	-	3/4	83	Post	No	0
wt	24	1.9	Ductal/NST	0/15	59	Post	Yes	-
wt	26	3.0	Ductal/NST	0/21	75	-	No	54
wt	27	2.5 (B)	Ductal/NST	-	57	Post	No	364
wt	32	3.9 (B)	Ductal/NST	0/5	47	Post	No	1
wt	33	2	Ductal/NST	10/17	50	-	No	71
wt	37	4.0	Squamous	-	66	Post	No	4
wt	40	7.5	Ductal/NST	4/7	49	-	No	1
wt	41	1.5	Ductal/NST	0/3	52	Post	Yes	-
wt	42	1.5	Ductal/NST	1/4	47	Peri	No	82
wt	45	3.8	Ductal/NST	0/2	51	Pre	No	0
wt	46	2.5	Tubular	2/5	59	Post	No	119
wt	49	1.6	Mucoid	3/3	39	Pre	No	23
wt	50	-	Ductal/NST	0/3	37	-	Yes	3
wt	53	2.2	Ductal/NST	5/9	54	Post	No	150
wt	54	2.8	Ductal/NST	-	55	Post	No	186
wt	55	3.3	Ductal/NST	0/14	67	Post	No	0
wt	56	2.0	Ductal/NST	0/1	75	Post	No	204
wt	58	1 (B)	Ductal/NST	1/6	57	-	No	-
wt	59	2.7 (B)	Ductal/NST	1/5	61	Post	No	536
wt	60	2.2 (B)	Ductal/NST	-	59	Post	No	392
wt	62	- (B)	Ductal/NST	-	54	Post	No	371
wt	63	3.8	Ductal/NST	0/3	33	Pre	No	44
wt	65	2	Scirrhous	3/3	45	Post	No	76
wt	68	1.5	Ductal/NST	0/8	52	-	No	212
wt	69	0.8	Ductal/NST	0/8	52	-	No	199
wt	70	1.2	Ductal/NST	0/2	50	Post	No	-
wt	74	- (B)	-	-	62	Post	No	240
wt	75	1.9	Ductal/NST	0/7	51	Post	No	3
wt	76	3.5	Mucoid	1/10	52	Post	No	62
wt	79	1.8 (B)	Ductal/NST	3/15	33	Pre	No	7
wt	81	-	Ductal/NST	18/19	64	Post	Yes	230
wt	83	2.8	Ductal/NST	2/3	47	Pre	No	36
wt	86	3.0	Ductal/NST	0/21	42	-	No	51
wt	87	2.5	Ductal/NST	0/21	42	-	No	37
wt	89	1.4 (B)	Lobular variant	0/4	39	Pre	No	35
wt	90	1.4	Ductal/NST	1/1	56	Post	No	243
wt	91	3.2	Ductal/NST	1/11	33	Pre	No	0
wt	94	2.8	Ductal/NST	0/12	60	Post	No	268

Table 6.1 (continued)

p53 gene	Tumour Number	Size (cm)	Histological Tumour Type	Nodal Status	Patient Age	M/P Status	Family History	ER level (pg)
wt	99	2.9	Ductal/NST	0/4	59	Post	No	277
wt	100	3.8	Tubular	0/12	48	Pre	No	88
wt	106	1.5	Ductal/NST	0/4	40	-	No	21
wt	107	2.3	Tubular	3/8	64	Post	Yes	149
wt	108	1.7	Ductal/NST	0/14	64	Post	Yes	321
wt	109	2.1	Ductal/NST	0/3	56	Post	No	75
wt	112	1.8	Papillary	0/10	58	-	No	232
mut	8	3.2	Ductal/NST	1/6	65	Post	No	122
mut	10	2.8	Ductal/NST	0/10	50	-	No	21
mut	11	2.0	Ductal/NST	10/17	50	-	No	3
mut	12	3.4	Ductal/NST	10/17	50	-	No	4
mut	18	2.8	Ductal/NST	3/10	79	Post	No	0
mut	21	3.5	Ductal/NST	7/10	66	-	No	0
mut	25	5.5	Lobular variant	5/10	70	Post	No	0
mut	34	2.2 (B)	Ductal/NST	0/4	66	Post	No	8
mut	57	1.9	Ductal/NST	4/4	66	Post	No	79
mut	61	2.6 (B)	Ductal/NST	-	62	Post	No	225
mut	64	1.7	Ductal/NST	1/3	50	Peri	No	1
mut	67	2.2	Ductal/NST	0/4	50	-	No	92
mut	71	1.3 (B)	Ductal/NST	1/1	47	Post	No	436
mut	72	1.7 (B)	Ductal/NST	0/3	56	Post	No	658
mut	84	4.5	Ductal/NST	3/5	55	Post	No	0
mut	85	5.5	Ductal/NST	0/21	42	-	No	4
mut	88	3.5	Ductal/NST	1/4	41	-	No	1
mut	92	1.2	Ductal/NST	0/5	38	Peri	Yes	61
mut	95	2.0	Ductal/NST	-	87	Post	No	11
mut	98	3.8	Ductal/NST	0/12	75	Post	No	0
mut	101	3.5	Comedo	0/12	59	Post	No	0
mut	110	1.2	Ductal/NST	0/4	65	Post	Yes	570
mut	113	-	Medullary	0/4	35	-	No	9
mut	114	1.4	Ductal/NST	0/12	36	Post	Yes	4

NST - No Special Type
(B) - Biopsy
M/P - menopausal
wt - wild type
mut - mutant
ER - oestrogen receptor

Table 6.2 Pathological, clinical and molecular data on the breast cancer patients and tumours.

LOH at p53	Tumour Number	Size (cm)	Histological Tumour Type	Nodal Status	Patient Age	M/P Status	Family History	ER level (pg)
L	14	1.4	Ductal/NST	1/17	44	Peri	Yes	51
L	15	3.0	Ductal/NST	1/17	44	Peri	Yes	39
L	16	2.0 (B)	Ductal/NST	-	60	-	Yes	80
L	18	2.8	Ductal/NST	3/10	79	Post	No	0
L	21	3.5	Ductal/NST	7/10	66	-	No	0
L	34	2.2 (B)	Ductal/NST	0/4	66	Post	No	8
L	35	1.2 (B)	Tubular	0/3	66	Post	No	275
L	40	7.5	Ductal/NST	4/7	49	-	No	1
L	42	1.5	Ductal/NST	1/4	47	Peri	No	82
L	57	1.9	Ductal/NST	4/4	66	Post	No	79
L	59	2.7 (B)	Ductal/NST	1/5	61	Post	No	536
L	72	1.7 (B)	Ductal/NST	0/3	56	Post	No	658
L	79	1.8 (B)	Ductal/NST	3/15	33	Pre	No	7
L	91	3.2	Ductal/NST	1/11	33	Pre	No	0
L	99	2.9	Ductal/NST	0/4	59	Post	No	277
L	100	3.8	Tubular	0/12	48	Pre	No	88
L	101	3.5	Comedo	0/12	59	Post	No	0
NL	19	1.2	Carcinoid	3/10	79	Post	No	-
NL	20	1	Ductal/NST	0/3	72	Post	No	-
NL	24	1.9	Ductal/NST	0/15	59	Post	Yes	-
NL	26	3.0	Ductal/NST	0/21	75	-	No	54
NL	29	1.8 (B)	Ductal/NST	2/4	34	Pre	No	36
NL	37	4.0	Squamous	-	66	Post	No	4
NL	45	3.8	Ductal/NST	0/2	51	Pre	No	0
NL	63	3.8	Ductal/NST	0/3	33	Pre	No	44
NL	65	2	Scirrhous	3/3	45	Post	No	76
NL	74	- (B)	-	-	62	Post	No	240
NL	75	1.9	Ductal/NST	0/7	51	Post	No	3
NL	85	5.5	Ductal/NST	0/21	42	-	No	4
NL	86	3.0	Ductal/NST	0/21	42	-	No	51
NL	90	1.4	Ductal/NST	1/1	56	Post	No	243
NL	93	1.4	Ductal/NST	1/4	57	-	No	119
NL	103	2.5	Papillary	6/12	68	Post	No	195
NL	107	2.3	Tubular	3/8	64	Post	Yes	149
NL	108	1.7	Ductal/NST	0/14	64	Post	Yes	321

NST - No Special Type
(B) - Biopsy
NL - No LOH, L - LOH
M/P - menopausal
ER - oestrogen receptor

Table 6.3 Association between p53 mutation and clinical pathological parameters

Clinical Parameter	Number of tumours with p53 mutation	Number of tumours without p53 mutation	Total number of tumours	Test	P value
Tumour size:					
< 2.0 cm	5	15	20	χ^2	0.75 > P > 0.5
≥ to 2.0 cm	14	26	40		NS
Nodal Status:					
Positive	11	21	32	χ^2	P > 0.9
Negative	11	25	36		NS
Patient Age:					
<50	6	18	24	χ^2	0.5> P > 0.25
≥ 50	18	36	54		NS
Menopausal Status:					
Pre/Peri	2	12	14	F.E.	0.01 > P > 0.001
Post	14	29	43		Considered NS due to paucity of data (section 6.3)
Family History:					
Yes	3	9	12	F.E.	0.9 > P > 0.75
No	21	45	66		NS
Oestrogen Receptor					
≤ 20	14	11	25	χ^2	0.05 > P > 0.01
> 20	9	37	46		

χ^2 - chi squared test
F.E. - Fisher exact test
NS - not significant

Table 6.4 Association between LOH at the p53 locus and clinical pathological parameters

Clinical Parameter	Number of tumours with LOH	Number of tumours without LOH	Total number of tumours	Test	P value
Tumour size:					
< 2.0 cm	3	7	10	F.E.	0.75 > P > 0.5
≥2.0 cm	8	9	17		NS
Nodal Status:					
Positive	10	7	17	χ^2	0.5> P > 0.25
Negative	6	9	15		NS
Patient Age:					
<50	7	5	12	χ^2	0.5 > P > 0.25
≥50	10	13	23		NS
Menopausal Status:					
Pre/Peri	6	3	9	F.E.	0.5 > P > 0.25
Post	8	11	19		NS
Family History:					
Yes	3	3	6	F.E.	P > 0.9
No	14	15	29		NS
Oestrogen Receptor:					
≤20	7	4	11	χ^2	0.5> P > 0.25
> 20	10	11	21		NS

χ^2 - chi squared test
F.E. - Fisher's Exact test - chi square test
NS - not significant

6.3 Discussion

The association of p53 mutation and menopausal status ($0.01 > P > 0.001$) could reflect a greater incidence of p53 mutations in tumours from post-menopausal patients. However it is likely that it is a consequence of the paucity of information on menopausal status in the set of patients presenting with tumours containing a p53 mutation (Table 6.1). Information on menopausal status was available for 16/24 of these patients. Of the remaining 8 patients only one patient was aged over 50 years suggesting a lack of information on menopausal status among patients under 50. p53 mutation was associated with low levels of oestrogen receptor protein ($0.05 > P > 0.01$). As oestrogen negative tumours have a poor prognosis a relationship between p53 mutation and aggressive tumour behaviour is suggested.

Despite the number of groups that have characterised p53 mutations in breast tumour samples only two have attempted to correlate p53 mutation with pathological/clinical parameters. One study used 60 breast tumour samples, 16 of which had p53 mutations (Thompson *et al.*, 1992). Significant association was found between p53 mutation and low levels of oestrogen receptor protein ($p < 0.01$) but not with tumour size, stage, nodal status, menopausal status or family history. The other study analysed 96 breast cancer patients, 18 of which had presented with tumours containing a mutant p53 gene (Mazars *et al.*, 1992b). Mutation of the p53 gene was associated with low levels of oestrogen ($P = 0.044$) and progesterone receptor protein ($P = 0.012$) but not with tumour grade, nodal status or patient age. The results of the current study confirm the findings of Mazars *et al.* (1992b) and Thompson *et al.* (1992) as a low level of oestrogen receptor protein was the only parameter to show significant association with p53 mutation ($0.05 > P > 0.01$).

Considering the amount of effort in identifying and characterising p53 mutations at the DNA level it is perhaps not surprising that efforts to assign a prognostic role to p53 have concentrated on immunohistochemical detection of the over-expressed protein. In total 10 studies have attempted to correlate pathological/clinical factors to p53 over-expression. Initial studies estimated the extent of antibody staining in 40-200 breast tumour samples (section 5.3) and found significant associations with a number of parameters (Cattoretti *et al.*, 1988; Davidoff *et al.*, 1991c; Horak *et al.*, 1991; Koutselini *et al.*, 1991; Ostrowski *et al.*, 1991; Walker *et al.*, 1991). In

concordance with the present study the majority of investigators found a statistically significant association with low levels of oestrogen receptor protein as well as with other pathological/clinical parameters associated with poor prognosis such as high tumour grade and axillary lymph node involvement.

Consequently the association of p53 over-expression and prognosis was investigated in four large studies. The study by Poller *et al.* (1992) analysed 146 breast tumour samples and found a non-significant trend between p53 over-expression and poor patient survival. A larger study by Thor *et al.* (1992), using 589 breast tumour samples, found a significant association with shorter metastasis free survival and overall survival which was confirmed in both lymph node negative and lymph node positive tumour populations. The two remaining studies investigated the potential of p53 over-expression for the identification of ANN patients with a high risk of tumour recurrence. A significant association with p53 over-expression and overall survival was found in 289 patients in a study by Isoba *et al.* (1992). However proliferation rate and tumour size, but not p53 over-expression, were shown to be independent predictors of survival. In contrast, a recent study by Allred *et al.* (1993) grouped 700 breast tumour samples into three classes based on antibody staining: non-expressors; low expressors; and high expressors. The study found significant differences between all groups when considering reduced disease free survival and between non-expressors and high expressors when considering overall survival. Over-expression of the p53 protein was also found to be an independent predictor of poor prognosis despite a high correlation with tumour proliferation rate.

Despite attempts to link p53 mutation at the DNA level to pathological parameters, the association with tumour recurrence or overall survival has not been determined (Mazars *et al.*, 1992b; Thompson *et al.*, 1992). Data on patient survival is currently being gathered to investigate the usefulness of this genetic alteration as an indicator of prognosis. Since the detection of p53 mutations at the DNA level is both labour intensive and time consuming a major effort would needed to analyse the p53 gene in numbers equivalent to those used in the immunohistochemical studies (Thor *et al.*, 1992; Isoba *et al.*, 1992; Allred *et al.*, 1993). In practical terms alone the determination of p53 mutation at the protein level is a much simpler and more straightforward process. However,

despite encouraging results much confirmatory work, in the form of studies standardised for methods, patient populations and therapeutic treatments, needs to be undertaken before p53 over-expression can be used as a disease marker in a clinical setting (Thor *et al.*, 1993).

Chapter 7
Discussion

7.1 Summary.

The experiments detailed in this thesis are a continuation of the work of Mackay *et al.* (1988a) who observed a high rate of LOH at loci near the telomere of chromosome 17p in matched control/breast tumour pairs. Using material from the same tumour bank the rates of LOH occurring at other loci on chromosome 17p were investigated with two aims in mind. First to determine whether the high frequency of LOH observed at the 17p13.3 locus extended to other more centromeric loci. Secondly, due of the proximity of two of the probes used to the tumour suppressor gene p53, to determine what role, if any, the p53 gene had in breast cancer development. Results from the LOH studies revealed that the probes mapping near to the p53 locus showed a LOH frequency of 49%. Furthermore two breast tumours were identified which showed LOH at 17p13.1, the site of the p53 gene, but retained heterozygosity at loci at 17p13.3, implicating the p53 gene in the development of these two tumours.

Consequently all the breast tumour DNAs were examined for p53 mutations in exons 5-9 of the gene using the HOT mutation detection technique and it was estimated that approximately 40% of breast tumours contained a mutant p53 gene. Most breast tumours with p53 mutations showed accompanying LOH although many tumours with LOH had an apparently normal remaining p53 allele. Possible explanations for this observation include inactivation of the p53 protein by mechanisms other than mutation in exons 5-9, failure of the HOT technique to detect all mutations and the presence of additional tumour suppressor genes on chromosome 17. Somatically acquired p53 mutations were found in 19 codons in exons 5, 6, 7, 8, and 9. Analysis of breast cancer p53 mutations detected in this and other studies revealed a preponderance of mutations at guanine residues, suggesting a role for exogenous carcinogens in breast tumorigenesis. A preponderance of mutations at codons 175, 194, 245, 248, 273, 280 and 285 was found but no particular hotspot was identified. p53 mutation was shown to be associated with low oestrogen receptor levels but not with patient age, nodal status, family history, tumour size. LOH at loci near the p53 gene was not associated with any pathological/clinical parameter.

7.2 Future studies.

The major problem with the present study, and one which is common with many studies using breast tumour material, is that the cellular composition of the samples themselves is unknown. Few studies using breast tumour tissue have undertaken to ensure that either the material analysed is composed of tumour cells only or that the extent of normal cell contamination within the samples is known (Lundberg *et al.*, 1987; Devilee *et al.*, 1990; Larsson *et al.*, 1990). The ability to isolate pure tumour cell populations would be invaluable for the molecular analysis of breast cancer. Methods have been devised to isolate malignant cells from normal ones by immunological selection but these techniques are costly and produce a yield of only 10% (Linehan *et al.*, 1989).

A method that offers insight into the genetic alterations occurring in breast tumour samples at a new level is the touch preparation method devised by Kovach *et al.* (1991). Using this method clusters of malignant cells were able to be isolated from frozen or fresh breast tumours containing a mixture of malignant and non-malignant tissue. Successful PCR amplification of tumour DNA from as few as 10 malignant cells was achieved and mutations of the p53 gene were detected and analysed without the need to consider the possibility of normal cell contamination. An addition benefit of this technique is that isolating cells from the same part of the tumour increases the chances of obtaining cells originating from the same clone. The possibility of tumour heterogeneity occurring within the group of analysed cells is therefore reduced as cells within a cluster would be expected to share the same genetic alterations.

By using a technique that can identify genetic alterations in breast tumour samples without the need to consider normal cell contamination or tumour heterogeneity, the relationship between p53 mutation and the loss of the remaining wild type allele can be investigated properly. Breast tumours which show p53 mutation without accompanying loss of the wild type allele have been found at a low frequency in a number of studies, including this one. This observation however may simply be a result of either normal cell contamination or tumour heterogeneity within the analysed sample (Mazars *et al.*, 1992b). Studies using touch preparations offer the possibility of confirming the identification of breast tumour samples showing p53 mutation without LOH. Of the 16 mutations detected in breast tumour cells using this technique only one, a deletion of 3 base pairs, showed the

presence of the normal allele on sequencing. This suggests that mutation of a single copy of the p53 gene can offer a selective advantage during breast tumorigenesis. However as 15/16 breast tumours with a mutant p53 allele had loss of the accompanying wild type allele the importance of the removal of both copies of the gene in the majority of breast tumours was demonstrated (Kovach *et al.*, 1991; Sommer *et al.*, 1992).

The ability to isolate pure tumour cell populations would greatly improve both the analysis of other tumour suppressor genes and the quality of LOH mapping data produced in future studies in breast cancer. Normal cell contamination and tumour heterogeneity within the majority of tumour samples in this study prevented the production of useful mapping data. The masking of LOH by normal cell contamination has also been demonstrated in experiments using immunologically selected renal carcinoma cells (Linehan *et al.*, 1989). In this analysis the three informative samples studied all showed little or no reduction in relative allele intensity before tumour cell selection. After selection a greater than 90% reduction in allele intensity was observed. Despite this, any LOH analysis using malignant cells isolated using this method would still have to take into account the possibility of tumour heterogeneity.

The touch preparation technique is capable of analysing tumour tissue without the need to consider the effects of either normal cell contamination or tumour heterogeneity although it does not yield enough DNA to allow LOH assays by the southern blotting technique. However, the detection of LOH using PCR markers is now possible and has been successfully used in lung, bladder, renal and breast carcinomas (Merlo *et al.*, 1991; Oka *et al.*, 1991; Ganly *et al.*, 1992). As when using the southern blotting LOH assay, only those tumours containing less than 25-30% normal cell contamination can be analysed visually (Vogelstein *et al.*, 1988; Merlo *et al.*, 1991). The use of both PCR markers for the detection of LOH and the touch preparation technique would enable visual evaluation of LOH data, without the need for quantitative densitometry, and could be carried out using very small amounts of DNA, so helping to conserve valuable tumour samples. LOH studies using PCR markers would also be less labour intensive, less expensive and less time consuming than those based on conventional southern blotting techniques and would facilitate rapid identification of tumour samples offering potential mapping information for the isolation of tumour suppressor genes.

Investigation of LOH in this and other studies has previously depended on the use of RFLP markers mapping up to several megabases away from the gene of interest. For example, YNZ22 is commonly used to determine LOH at the p53 locus, 20 megabases away (Y. Nakamura, personal communication). Intragenic polymorphisms identified though the study of a particular gene can be easily used as allelic markers by PCR thus enabling LOH to be determined at the site of interest. A PCR marker based on the codon 72 CGC>CCC polymorphism in the p53 gene has been used successfully in the analysis of bladder and renal carcinomas (Merlo *et al.*, 1991; Oka *et al.*, 1991) and additional polymorphism at codons 21 (Ahuja *et al.*, 1990) and 72 (Harris *et al.*, 1986) and in introns 1 (Ahuja *et al.*, 1989) and 6 (Prosser and Condie 1991) could also be utilised. PCR primers identifying the YNZ22/D17S5 locus (Horn *et al.*, 1989; Gecz 1991) have been determined and have enabled rapid identification of deletions in the Miller-Dieker syndrome (Batanian *et al.*, 1990). These could potentially be of great use in the mapping of the putative 17p13.3 tumour suppressor gene in breast cancer.

These types of technique have the potential to investigate the extent of molecular changes within breast tumour samples and the relationship between genetic alteration and tumour characteristics. The high frequency of tumour heterogeneity observed in LOH studies (Larsson *et al.*, 1990; Chen *et al.*, 1992) suggests the evolution of different clones within the same tissue occurs in the majority of tumours. The touch technique could be used to analyse directly cells originating from different areas of a single tumour for several genetic alterations in order to determine both the extent of heterogeneity and the clonal relationship between different cell populations. In tumours where more than one type of clone is found it would be of great interest to try and identify which clones are capable of metastasising to axillary lymph nodes and distant organs. In this way genetic changes important in the metastatic process, which may have potential as prognostic markers, could be identified.

In a similar way the clonal relationship between the carcinoma in situ and invasive carcinoma stages could be examined in tumours where both components are present. A definite clonal relationship between the two stages could be established and the genetic changes important in determining invasive characteristics identified. Paralleling the study by Davidoff *et al.* (1991b)

tumours containing both carcinoma in situ and invasive carcinoma cells could be used to determine whether the mutation of the p53 gene occurs before or after invasive properties are acquired. Mutations occurring only in the invasive components of such tumours could be regarded as late events while mutations present in both components could be considered as alterations occurring earlier in the development of the tumour. A temporal ordering for p53 mutation and loss of the remaining wild type allele could be determined by examining both these alterations in pure tumour cell populations from a single tumour. Additionally, in tumours showing more than one histological pattern, investigation at the tumour cell level could be used to identify genetic changes important in determining histological differentiation.

The majority of these proposed studies require careful analysis of breast tumour samples at a histological level. The breast tumour bank used in this current study has no data of this type and so new tumour material would need to be collected and analysed before these experiments could be attempted. Although the current tumour bank has been shown to be unsuitable for the generation of mapping data it has been successfully used for the identification of mutations in the p53 gene (Coles *et al.*, 1992). As previously discussed, the p53 gene mutation spectrum observed in breast tumours does not indicate any specific factor involved in the mutagenesis of the p53 gene. It is likely that the p53 mutation spectrum is a product of several different factors and continuation of work of this type may not be particularly productive. However breast tumours show LOH at a high frequency at a number of loci and further mutation analysis experiments on newly identified tumour suppressor genes could be carried out using material from the breast tumour bank. For example putative tumour suppressor genes residing on 1q, 3p, 6q, 17p, 17q and 18q could be analysed both to confirm their role in breast tumorigenesis and to identify the types of alteration occurring within these genes. Since analysis of the p53 mutation spectrum has not produced information in the way that a similar analysis in hepatocellular carcinoma and squamous carcinoma of the skin has done, by analysing a number of tumour suppressor genes in breast tumour samples it may be possible to identify a genetic alteration which has the potential to indicate a specific factor involved in the development of breast cancer.

In this study 79 sporadic primary breast tumours were successfully screened using the HOT technique for mutations in the p53 gene. However, despite the advantage of a 100% detection rate and the ability to confirm mutations by the modification and cleavage pattern, the technique is time consuming, labour intensive and requires the use of hazardous chemicals. Any further mutation studies on other tumour suppressor genes using the current tumour bank would be probably be more effectively carried out using the SSCP technique. As well as having a high mutation detection rate it is rapid and less costly than either the HOT or DGGE/CDGE techniques, making it ideal for the scanning of a large number of tumour samples. Unfortunately the less than 100% detection rate poses problems when using the technique for mutation analysis as potential mutation hotspot sites could remain undetected. This can be overcome by using overlapping SSCP fragments, so that, in effect, each nucleotide base pair is screened twice, or by analysing a subset of the samples with the HOT technique. Sufficient DNA from the breast tumours screened for p53 mutations remains for similar mutation studies to be carried out. This makes the current tumour bank an excellent resource for determining the frequency and types of mutation present in other tumour suppressor genes in breast cancer. In addition, once information on patient outcome has been collected, such studies could indicate the potential for newly discovered tumour suppressor genes as prognostic markers in the clinical management of the disease.

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Abbreviations

A	adenine
Arg	arginine
ATP	adenosine triphosphate
AMPS	ammonium perisulphate
ANN	axillary node negative
APC	adenomatous polyposis coli
BDPE	benzo(a)pyrene diolepoxide
bp	base pair
BSA	bovine serum albumin
C	cytosine
CDGE	constant denaturing gel electrophoresis
cDNA	complementary deoxyribonucleic acid
Cys	cystine
dATP	deoxyadenosine triphosphate
DCC	deleted in colon cancer
dCTP	deoxycytidine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytidine triphosphate
ddGTP	deoxyguanidine triphosphate
ddTTP	deoxythymidine triphosphate
DGGE	denaturing gradient gel electrophoresis
dGTP	deoxyguanidine triphosphate
DNA	deoxyribonucleic acid
ds	double strand
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetra-acetic acid
ER	oestrogen receptor
G	guanine
Gly	glycine
HA	hydroxylamine hydroxide
HBC	hereditary breast cancer
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCl	hydrogen chloride
HGU	Human Genetics Unit

His	histidine
HOT	amplification and mismatch detection
Ile	isoleucine
kb	kilobase pairs
kD	kilodalton
LFS	Li-Fraumeni syndrome
LOD	log of difference
LOH	loss of hetrozygosity
MCC	mutated in colon cancer
MDM2	murine double minute 2
MRC	Medical Research Council
mRNA	messsenger ribonucleic acid
NaCl	sodium chloride
NaOH	sodium hydroxide
NF	neurofibromatosis
NSCLC	non-small cell lung cancer
OsO_4	osmium tetroxide
PCR	polymerase chain reaction
Phe	phenylalanine
PR	progesterone receptor
Pro	proline
Rb	retinoblastoma
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
SCLC	small cell lung cancer
SDS	sodium dodecyl sulphate
Ser	serine
ss	single strand
SSC	squamous cell carcinoma of the skin
SSCP	single strand conformation polymorphism
SRO	smallest region of overlap
T	thymidine
TEMED	N, N, N', N'-tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethan
Trp	tryptophan
UV	ultra violet
V	volts

Val	valine
VNTR	variable number of tandem repeats
WT	Wilm's tumour

Published Papers

Coles C., Thompson A.M., Elder P.A., Cohen B.B., Mackenzie I.M., Cranston G., Chetty U., Mackay J., Macdonald M., Nakamura Y., Hoyheim B. and Steel C.M. (1990) Evidence implicating at least two genes on chromosome 17p in breast carcinogenesis. Lancet **336**: 761-763.

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MEDICAL SCIENCE

Evidence implicating at least two genes on chromosome 17p in breast carcinogenesis

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The DNA of paired tumour and blood leucocyte samples from a large series of breast cancer patients was analysed to map regions of loss of heterozygosity on chromosome 17. The high frequency of loss of heterozygosity on 17p was confirmed, and a third of informative tumours had also lost an allele at the long arm locus THH59. On the short arm two distinct regions of loss of heterozygosity were identified, in bands p13–3 and p13–1. The latter probably involves the structural gene p53, which has been implicated as an oncogene or as a tumour suppressor in various human cancers. 17p 13–3, however, showed a significantly higher frequency of loss of heterozygosity, and there was no correlation between allele losses at the two sites. Nevertheless, loss of heterozygosity at 17p 13–3 is associated with overexpression of p53 mRNA, suggesting the existence of a gene some 20 megabases telomeric of p53 that regulates its expression. Lesions of this regulatory gene seem to be involved in the majority of breast cancers.

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Introduction

Loss of heterozygosity at specific loci in tumour tissue may indicate deletion of tumour suppressor genes.[1] Several sites of loss of heterozygosity have been reported in human breast cancer, particularly on the short arm of chromosome 17.[2-4] The same chromosome arm shows loss of heterozygosity in small-cell lung cancer,[5] colon cancer,[6] ovarian cancer,[7] osteosarcoma,[8] and astrocytoma.[9] The occurrence of "cancer families" showing clusters of two or more of these disorders also suggests that the different tumour types share at least one gene that when inactivated can initiate malignant change. p53 is a strong candidate since it is located on chromosome 17p, is overexpressed, mutated, or both in at least a proportion of the tumours examined and can function as a tumour suppressor, or when mutated, as an oncogene.[9-11] We now report the distribution of loss of heterozygosity at multiple loci on chromosome 17 in breast cancer.

Patients and methods

Fresh tumour tissue was obtained at operation from 168 patients with breast cancer attending the Edinburgh breast unit. Most had lesions less than 5 cm in diameter detected by self-examination or through the breast cancer screening programme. A few patients had received tamoxifen, but no other treatment had been given before surgery. A venous blood sample was taken from each patient, with informed consent, and high molecular weight DNA was extracted from tumour and whole blood by a conventional phenol/chloroform procedure. Samples of DNA were digested with restriction enzymes according to the manufacturers' instructions. Gel electrophoresis and Southern blotting were carried out[2,12] with blood and tumour DNA from the same patient in adjacent tracks and control (placental or tonsil) DNA of known genotype in the outer tracks of each gel. The probes used and their map locations are given in table I. They include pBHP53, derived from a cosmid library of genomic p53.[14] Because of the need to conserve DNA, particularly from the smallest tumour biopsy samples, we could not analyse every tumour with every probe. However, this series is the largest of breast tumours and the most comprehensive mapping of loss of heterozygosity on chromosome 17 yet reported.

Results

The highest frequency of loss of heterozygosity (58%) was detected by the probe YNZ22 (table I), in accord with previous reports.[2-4,12] For all the other loci examined on 17p the rates of allele loss were significantly lower than that for

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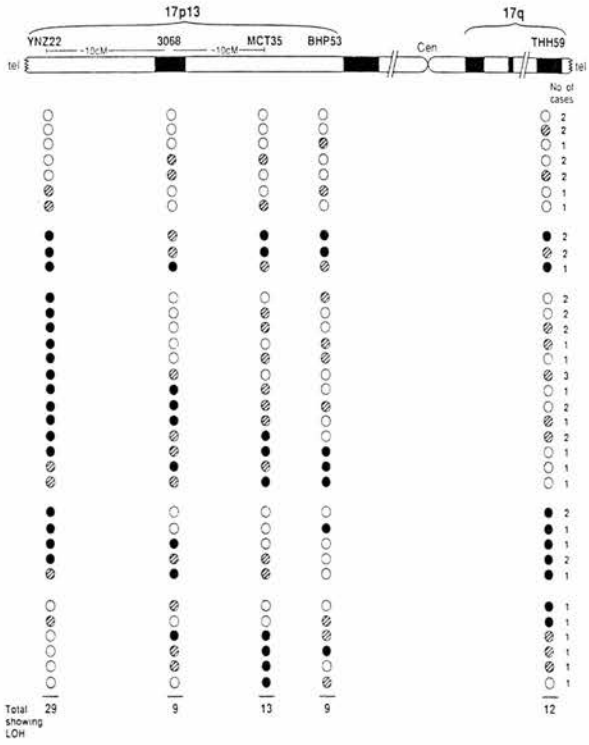
TABLE I—LOSS OF HETEROZYGOSITY AT LOCI ON CHROMOSOME 17

Locus	Probe[13,14]	Map location	Restriction enzyme	No of tumours		No (%) of tumours with LOH
				Analysed	Informative	
D17S30	YNZ22	p13.3	*Taq* I	142	98	57 (58%)
	C3068*	p13.2	*Hae* III	81	51	17 (33%)
D17S31	MCT35.1	p13.1	*Msp* I	113	54	24 (44%)
p53	pBHP53	p13.1	*Bam* HI	138	81	22 (27%)
D17S4	pTHH59	q23–25.3	*Taq* I	67	47	16 (34%)

* = Y. Nakamura, unpublished.
LOH = loss of heterozygosity.

YNZ22 ($p < 0.01$, chi-square test) showing that the region of 17p most commonly deleted in human breast cancer does not include the structural gene for p53. Nevertheless, the frequency of allele losses at the other 17p loci was much higher than that at randomly chosen sites on other chromosomes.[2,3] There was a similar frequency of loss of heterozygosity (34%) at the long arm locus D17S4; this rate is higher than we had reported previously and than that found by two other groups with different 17q probes.[3,15] Our earlier series of breast tumours[2] forms a part of this larger collection. The original autoradiographs were re-examined and seven of the tumour/blood pairs were subjected to reanalysis with probe pTHH59, on fresh samples of DNA. In no instance was our previous interpretation changed. The change in the overall rate is therefore simply a consequence of increasing the size of the population sampled.

When the data were tested by pairwise comparisons for concordance of allele loss at any two loci (table II), it was clear that loss of heterozygosity at YNZ22 was independent of loss at any of the other loci. The status of MCT35.1 correlated significantly with that of pBHP53 and of C3068, whereas allele losses at the latter two loci were independent of each other. These findings support the map order suggested from multipoint linkage analysis—3068–MCT35.1–BHP53–cen. These observations are incompatible with any single shortest region of overlap for chromosome 17p deletions in this series of breast tumours. When the data were combined with a conventional plot of loss of heterozygosity pattern for those tumours informative at three or more of the loci (see accompanying figure) it was apparent that there must be at least two shortest regions of overlap on the short arm occurring independently and with different frequencies—one close to YNZ22 and the other close to MCT35.1. A deletion centred on either of these subregions can include C3068 by extension, in one case towards the centromere and in the other towards the telomere. However, apart from the few cases in which



Patterns of loss of heterozygosity on chromosome 17 in 49 tumours informative at three or more loci.

● = loss of heterozygosity (usually complete loss of one allele, otherwise > 50% reduction in relative intensity of one band on Southern blot, confirmed by laser densitometry); ○ = heterozygosity retained; ⊘ = uninformative (or not tested).

heterozygosity appears to be lost from the whole length of the chromosome (implying probable monosomy or isodisomy), allele loss of THH59 on the long arm of chromosome 17 seems to be a further independent event, suggesting the possibility of a third region of specific deletion.

Discussion

This pattern of allele loss is quite different from that recorded in other settings. For example, a large series of colon tumours analysed in Edinburgh showed a very clear single shortest region of overlap on the long arm of chromosome 5 with the peak frequency of loss of heterozygosity close to the *apc* locus.[16] The pattern also differs from those reported for chromosome 17 in other

TABLE II—PAIRWISE COMPARISONS IN TUMOURS INFORMATIVE AT BOTH LOCI*

—	3068			MCT35.1			BHP53			THH59		
	LOH+	LOH−	p	LOH+	LOH−	p	LOH+	LOH−	p	LOH+	LOH−	p
YNZ22												
LOH+	8	11	0·20	9	15	0·63	9	20	0·24	9	11	0·28
LOH−	1	6		6	10		4	18		3	8	
3068												
LOH+	..	..	..	3	1	0·012	2	5	0·29	3	5	0·55
LOH−	..	..	..	1	16		2	17		5	11	
MCT35.1												
LOH+	..	..	..	..	..	..	9	7	0·006	3	5	0·55
LOH−	..	..	..	..	..	..	1	13		5	6	
BHP53												
LOH+	..	..	..	..	..	..	..	..	..	3	3	0·54
LOH−	..	..	..	..	..	..	..	..	..	7	10	

*Fisher's exact test.

tumours. In osteosarcoma[8] and colon cancer[6] deletions span much of the short arm but give a single shortest region of overlap centred on MCT35.1 (D17S31), whereas ovarian cancer shows an even higher frequency of allele loss on the long arm (at THH59) than on the short arm, with no evidence as yet for separate clusters of deletions within 17p.[17]

The association between allele loss on 17p and mutation of the retained copy of p53, shown in several tumour types,[5,9,11] may require further scrutiny in the light of these findings. Analysis of a subset of this series of tumours has shown that p53 mutations can occur in association with various patterns of allele loss at loci on 17p and are not confined to those with demonstrable deletions close to p53 itself.[18] Furthermore, many deletions centred on MCT35.1 exclude the locus identified by pBHP53. However, the orientation of genomic p53 with respect to MCT35.1 is not known, and it is possible that deletions affecting MCT35.1 but sparing pBHP53 remove portions of p53 coding or regulatory sequences, inactivating that copy of the gene.

It is also interesting that allele losses at YNZ22 are correlated with overexpression of p53 mRNA.[12] Since the loci are some 20 megabases apart and since allele losses at the two sites are independent, we postulate a separate gene, close to YNZ22, the function of which is to regulate p53 expression. Lesions involving that regulatory gene constitute the commonest molecular changes in human breast tumours. This hypothesis, of course, does not detract from the importance of p53 in relation to breast cancer but introduces a new level of complexity into that relation.

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Antimicrobial actions of calcium binding leucocyte L1 protein, calprotectin

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The calcium binding L1 protein was found to inhibit growth of blood culture isolates of *Candida* spp and cerebrospinal fluid isolates of *Cryptococcus neoformans*. Minimum inhibitory concentrations (MIC) were 4–128 mg/l, and concentrations 2–4 times the MIC were fungicidal. Blood culture isolates of *Escherichia coli, Klebsiella* spp, *Staphylococcus aureus,* and *Staphylococcus epidermidis* had MIC values of 64–256 mg/l. Antibacterial activity was strongly influenced by the nature of the culture medium. In view of the biological activity of L1, the name calprotectin is proposed to describe this antimicrobial protein with calcium binding properties.

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Introduction

Human leucocyte protein L1 was first described in 1980,[1] is partly characterised,[2,3] and is a cytoplasmic component of neutrophils, mononuclear cells, and squamous epithelial cells.[4,5] This 36·5 kD molecule contains 13·3 kD heavy ($L1_H$) and 8·2 kD light ($L1_L$) polypeptide chains, each of which can bind two calcium ions. Its quaternary structure probably contains one $L1_L$ and two $L1_H$ chains. Although the function

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2. MOLECULAR LESIONS IN BREAST CANCER

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An essential feature of malignancy is that the malignant phenotype is passed from the parent cell to its progeny *ad infinitum*. This suggests that the fundamental lesion in malignancy resides in the DNA. Strong supporting evidence for this theory comes from DNA instability syndromes (for example, ataxia-telangiectasia, Gatti *et al.*, 1988), in which DNA repair mechanisms are defective with an associated increased risk of malignant disease and from inherited cancer syndromes such as retinoblastoma and familial adenomatous polyposis which behave as autosomal dominant conditions. Breast cancer also has a heritable component as discussed in the "Etiology" section of this Supplement.

Support for this molecular hypothesis of breast cancer has been derived from cytogenetic and molecular techniques. Despite the difficulties encountered in cytogenetic analysis of solid tumours, aberrations have been demonstrated on chromosomes 1, 3, 5, 6, 10, 11, 12 and 17 (Ferti-Passantonopoulou and Panani, 1987); a particular association is reported with breakpoints on chromosome 1 at position q21 (Pathak and Goodacre, 1986).

Molecular lesions can be described under the headings of amplification, mutation, overexpression or deletion of genetic material. However, molecular studies can only target limited regions of the genome. The choice of where to begin the search may be based on a known oncogene (such as H-*ras*), a known functional gene (for example, the oestrogen receptor gene) or the site of a specific chromosomal abnormality. Alternatively, chance may provide the initial guide.

In breast cancer, several specific alterations in DNA or gene expression (mRNA or protein) in the tumour have been demonstrated. Somatic changes in tumour DNA fingerprints have been demonstrated using mini-satellite probes (Thein *et al.*, 1987), and surveys of gene amplification (Masuda *et al.*, 1987) and oncogene expression (Whittaker *et al.*, 1986) have led on to more detailed studies.

Oncogenes

A variety of experimental approaches have been used to detect genes capable of contributing to a malignant cellular phenotype in a dominant fashion at the cellular level, known as oncogenes. The normal human genome contains homologous sequences (proto-oncogenes) which can be activated in different ways to produce oncogenes.

The oncogene c-*myc* on chromosome 8 (q24) is amplified in 20–30% of primary breast cancers (Escot and Theillet, 1986; Varley *et al.*, 1987), and this is seen significantly more frequently in patients over 50 years of age at diagnosis (Escot and Theillet, 1986). In the original series no other association was found, but a later study identified amplified c-*myc* as a poor prognostic factor, being associated with early disease recurrence or death (Varley *et al.*, 1987). However, the significance of *myc* DNA amplification, as opposed to high levels of expression of the gene seen in up to 80% of breast cancer patients (Kozbor and Croce, 1984; Lee *et al.*, 1984), remains an unresolved question.

A better correlation between DNA amplification and level of gene expression has been found in studies on another oncogene, *erb* B-2 on 17q (Coussens *et al.*, 1985). Eighteen percent of primary tumours contain amplified *erb* B-2 (Slamon *et al.*, 1987), but 40% of patients who are lymph-node-positive at presentation have tumours with an amplified *erb* B2 gene (Slamon *et al.*, 1987) and in this subgroup there is a strong association between *erb* B-2 amplification, a shorter disease-free interval and early death (Slamon *et al.*, 1987, 1989; Wright *et al.*, 1989).

Another oncogene, *Int*2 on 11q13 (Casey *et al.*, 1986), is amplified in a small subset of tumours characterized by very aggressive growth leading to early death despite intensive chemotherapy (Zhou *et al.*, 1988). The current evidence therefore suggests that specific oncogene amplification occurs in a subgroup of primary tumours biologically more aggressive than the majority of breast cancers.

High levels of Ha-*ras*, a gene which encodes a protein p21 involved in signal transduction, have been found in the majority of breast tumours (Horan-Hand *et al.*, 1984). Although activating mutations of Ha-*ras* are probably rare in breast cancer (Rochlitz *et al.*, 1989), a concentration-dependent effect of p21 on tumorigenicity (Redmond *et al.*, 1988) and elevated Ha-*ras* mRNA levels in malignant compared to normal breast tissue (Spandidos and Agnatis, 1984; Slamon *et al.*, 1984) have been demonstrated. Ha-*ras* may be involved in the metastatic phenotype (Agnatis *et al.*, 1986; Lundy *et al.*, 1986). Certainly, in the experimental setting v-Ha-*ras* transfection induces oestrogen-independent tumorigenicity in previously oestrogen-dependent MCF-7 cells (Kasid *et al.*, 1987), suggesting that Ha-*ras* is involved in progression rather than initiation of breast cancer.

Other oncogene abnormalities have been reported less frequently. A rare c-*mos* polymorphism has been described

in breast cancer patients (Lidereau *et al.*, 1985); amplification of *myb* (Masuda *et al.*, 1987); overexpression of *fes* and *fms* (Slamon *et al.*, 1984) and elevated c-*fos* expression (Biunno *et al.*, 1988) are also recorded.

The concepts of tumour-suppressor genes and oncogenes are discussed elsewhere in this Supplement. It is sufficient to point out here that some genes (tumour-suppressor genes) appear to be involved in the inhibition of malignancy. The normal functions of such genes may be related to cell differentiation, growth control and genome stability. However, only one, the retinoblastoma gene, has been isolated and cloned to date and its function is still not entirely clear.

The use of highly polymorphic DNA probes to detect chromosomal lesions below the limit of visible resolution, but involving deletion or inactivation of particular genes, has permitted identification in primary breast tumour cells of at least 3 areas of the genome likely to be of fundamental importance. The first is on chromosome 11p. Allelic loss of the Harvey *ras* locus at 11p15 is found in 20–27% of human primary breast tumours (Mackay *et al.*, 1988*a*; Theillet *et al.*, 1986) and is reported to be associated with low oestrogen receptor status (Mackay *et al.*, 1988*a*; Lidereau, 1988) larger tumour size (Mackay *et al.*, 1988*a*) and high histological grade (Lidereau, 1988), suggesting that it could be taken as a feature of poor prognosis. However, more detailed examination of several loci on 11p has confused rather than clarified the picture in that it has not been possible to identify a single segment of the chromosome arm that is lost consistently. Lesions were found on 11p in about 50% of a small series of tumours fully characterized for 6 loci (Mackay *et al.*, 1988*a*).

The second potentially important locus is on 13q, which contains the first tumour-suppressor gene to be identified, the retinoblastoma (Rb) gene (Cavenee *et al.*, 1985). Forty percent of ductal tumours have allelic loss at 13q (Lundberg *et al.*, 1987), and rearrangements at the retinoblastoma locus have been found in 19% of primary breast tumours (Varley *et al.*, 1987) although the significance of these findings, in terms of prognosis for individual patients, remains unclear.

The third important locus is on 17p. Recent studies have demonstrated allelic loss at the locus identified by a highly polymorphic sequence YNZ22 in up to 60% of breast tumours (Mackay *et al.*, 1988*b*; Devilee *et al.*, 1989). Initially, no significant clinical or pathological associations were found (Mackay *et al.*, 1988*b*) but a larger series has identified a correlation between YNZ22 allelic loss and low oestrogen receptor status (Thompson *et al.*, 1990). These findings have been extended by studies on p53, a gene located on 17p (Isobe *et al.*, 1986). It can evidently behave either as an oncogene or a tumour suppressor, depending on whether its structure is normal or mutant (Green, 1989). p53 protein is also expressed in 15–25% of breast tumours (Cattoretti *et al.*, 1988), and a high level of p53 mRNA has been found in 30% of primary tumours (Thompson *et al.*, 1990). There is a significant association between a high level of p53 mRNA and YNZ22 allelic loss, raising the possibility that a p53-controlling element on 17p has been lost in some breast tumours.

Another important locus appears to be the ataxia-telangiectasia gene (11q22–24; Gatti *et al.*, 1988) since female carriers of the A-T gene have an increased risk of breast cancer (Swift *et al.*, 1987).

Failure to express oestrogen receptor has been associated with absence of an oestrogen-receptor DNA allele (Hill *et al.*, 1989) and an ER protein/ER mRNA ratio of >1.5 correlates with short time to relapse in ER-containing tumours (May *et al.*, 1989). The oestrogen-regulated mRNA pS2 (Masiakowski *et al.*, 1982) correlates closely with the effects of oestrogen on cell proliferation (Johnson *et al.*, 1989).

Multiple molecular lesions in individual tumours

The techniques used for defining changes at specific genetic loci are so much more sensitive than conventional cytogenetics that is has been possible to demonstrate a series of consistent DNA lesions present in many primary breast tumours. These lesions are very unlikely to be mutually exclusive, and it is probable that malignant cells accumulate 2, 3 or more of them in the course of tumour evolution as has already been demonstrated in colorectal cancer (Vogelstein *et al.*, 1989).

Thus, in breast cancer, it appears that some combination of oncogene over-activity (as demonstrated by activation, amplification or over-expression) and loss of tumour suppressor function (by mutation, deletion or reduced expression) occurs as the underlying mechanism for breast carcinogenesis. However, the number of lesions required, the order in which they occur and the relationship of these multiple steps to tumour progression and metastasis remain unknown.

Future studies

Identification of the breast cancer genes or DNA sequences

Indentification of the actual genes or DNA sequences of fundamental importance will come from more detailed mapping studies in tumours.

This is the approach that led to isolation of the Rb gene and is currently homing in on the adenomatous polyposis coli "*apc*" gene, implicated in colorectal cancer and located on chromosome 5q. If a particular locus is shown to

be lost in a high proportion of breast tumours, this will be very strong evidence that it is the site of a crucial tumour-suppressor gene. Careful analysis of tumours at different stages in the natural history of the disease from carcinoma *in situ* right through to metastatic deposits will be required to elucidate the sequence of molecular events that underlies initiation and progression of breast cancer.

A different approach, which can contribute to the mapping of "breast tumour genes" is the application of linkage analysis to families with a high incidence of breast cancer. A preliminary study on one large family in Edinburgh has recorded an inconclusive lod score (+1.8 at 0 recombination) for YNZ22, but no linkage was observed between heritable breast cancer and the Harvey *ras* gene. More extensive studies on 13q have also produced negative results but the major part of the human genome has yet to be surveyed.

Ultimately the question must be asked "what contribution can molecular analysis make to the prevention and/or treatment of breast cancer" The immediate prospect is that the prognosis for individual patients may be defined much more precisely and hence that the choice of aggressive or relatively conservative management may be made with more confidence. In the longer term we can envisage a much more profound understanding of the process of carcinogenesis which should open up the prospect of completely new approaches to treatment. It would, however, be misleading to suggest that gene therapy (*i.e.*, direct manipulation of the genome of malignant cells) will have a place in the foreseeable future. The problem of inducing a targeted change in the DNA of *every* tumour cell appears insurmountable, on the basis of current knowledge, and anything less would be bound to fail. It is more realistic to anticipate the identification of new biological pathways providing opportunities for specific and effective pharmacological intervention.

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SHORT COMMUNICATION

Constitutional p53 mutation in a non-Li-Fraumeni cancer family

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Two recent reports have described inherited mutations in p53 associated with the predisposition to develop early cancers (Malkin *et al.*, 1990; Srivastava *et al.*, 1990). These germ-line mutations were found in affected members of families with the Li-Fraumeni syndrome in which soft-tissue sarcomas in related children were associated with cancers of the breast and other organs among parents and relatives (Li & Fraumeni, 1969). In addition, a germ-line mutation in p53 has been identified in a 5-year-old patients with an intra-cranial malignancy and a strong family history of cancer (Metzger *et al.*, 1991). Finally, there are a further ten constitutional mutations reported for the Li-Fraumeni syndrome in a recently complied list of p53 mutations (Caron de Fromentel & Soussi, 1991). We now report a germ-line mutation of p53 in a non-Li-Fraumeni cancer family.

We detected a constitutional mutation in p53 while using the HOT technique (hydroxylamine/osmium tetroxide modification of mismatched basepairs (Cotton *et al.*, 1988; Prosser *et al.*, 1990, 1991)) to screen for alterations in this gene in a series of sporadic breast tumours. In one of these patients the p53 mutation was present in both the tumour and white blood cell DNA. This is an incidence of one in 136 patients, or 0.7%. Sequencing confirmed that both normal and mutant alleles were present in each sample. Interestingly, the tumour DNA showed loss of heterozygosity with the probe YNZ22 but no loss with pBHp53. The mutation is in exon 8 at codon 267 (Figure 1), changing arginine to glutamine, a basic to an uncharged amino acid. The mutation lies in the general region of previously published germ-line mutations in the p53 gene (amino acids 242–307, Malkin *et al.*, 1990; Srivastava *et al.*, 1990; Metzger *et al.*, 1991; Caron de Fromental & Soussi, 1991), although, unlike the majority of these mutations, it is not found in the conserved regions of the gene (one in region III, 13 in region IV and three in region V), but lies between conserved regions IV and V at an arginine codon which is invariant in all species studies (*Xenopus*, trout, chicken, rat, mouse, human) (Soussi *et al.*, 1989). The remaining codon at position 307 does not lie within a conserved region of the gene but is invariant in mammals. In data collated by Hollstein *et al.* (1991) which included 280 base substitutions distributed over 90 codons of the p53 gene, codon 267 was not reported mutated. In approximately 350 single base alterations in 93 codons collated by Caron de Fromentel and Soussi (1991) codon 267 was once reported mutated from CGG to CCG. The mutation we report is CGG to CAG.

Family studies and examination of medical records showed that the patient is indeed a member of a cancer family, but a family in which the age of onset of malignancy is not remarkably early (Figure 2). Information is available for a five generation pedigree in which it would appear that the constitutive mutation was either present five generations ago or arose as a germ-line mutation at that time. There are four recorded cancer deaths in the pedigree: breast cancer at age 53, breast cancer at age 67, lung cancer at age 66, ovarian cancer at age 63. The proband is alive with breast cancer at age 53. We have found the mutation in the proband, in her sister who is unaffected by cancer at age 37 years, and in a first cousin of the mother of the proband who is alive and unaffected by cancer at age 74 years. We have not been able to PCR archival material from the mother's lung tissue (which had been preserved in Bouin's fixative) and have therefore been unable to show the mutation in this woman who would appear to be an obligate carrier of the mutation.

Because the mutation is found in a 74 year old cancer-free relative of the proband, it would be difficult to argue that the mutation segregates with affected family members in the pedigree, while being absent from unaffected relatives. What we can say is that we have found a constitutive p53 mutation in the proband of a cancer family in which a variety of cancers have been noted over three generations. The mutation has been looked for and is present in two other family members, neither of whom has developed cancer. There are no cases of childhood or early cancer in this five generation pedigree. No member affected by cancer had remarkably early onset of the disease (at ages 42, 49, 53, 63 or 67 years), and, indeed, one member of the pedigree with the mutation remains unaffected in her 8th decade.

At the time of discovery of the Li-Fraumeni constitutive mutations, the median age of tumour development in affected family members was noted to be approximately 30 years and it was argued that the mutations were, from the cell's point of view, weak mutations (Vogelstein, 1990). Judging by the observed age of onset of disease in affected family members and by the absence of disease in a 74 year old carrier of the mutation, the mutation at codon 267 in this family is even weaker. It may be relevant to note that at least one parent and one grandparent in the six families originally reported to possess a constitutional p53 mutation were themselves obligate carriers of the respective mutations but had not developed cancer (Malkin *et al.*, 1990).

It may be expected that mutations in p53 which are compatible with viability and normal early development must be 'weak' mutations which confer only a small growth advantage to the cell and which cannot act as dominant negative mutations. On the other hand, no such constraint is imposed on the somatically acquired mutations in tumours which might be expected to show a greater variation and include

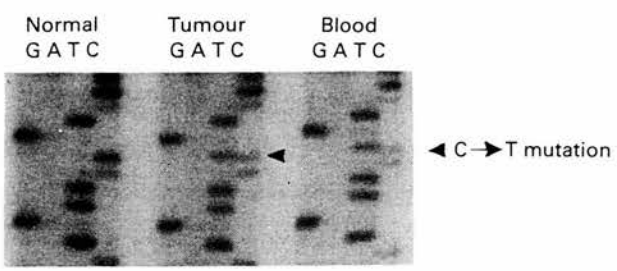

Figure 1 Sequence of the mutation.

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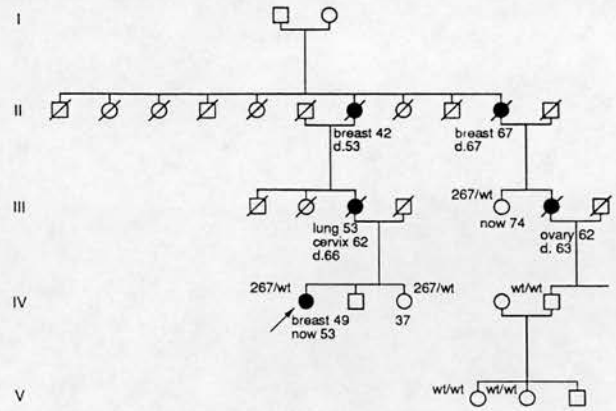

Figure 2 Five generation pedigree showing incidence of cancer (black circles). Numbers below circles represent age at which cancer was diagnosed, age at death, current age, as appropriate. Arrow indicates the proband. Individuals tested for the p53 mutation are shown as wt/267 (carriers) and wt/wt (non-carriers).

'strong' mutations conferring considerable growth advantage. There is evidence (Milner & Medcalf, 1991) for the 'strength' of only one of the constitutionally mutated codons, 248, where a CGG->TGG mutation does not behave in a dominant-negative way when co-translated *in vitro* with wild-type p53 protein. This contrasts with the *in vitro* demonstration of the dominant-negative effect of proteins carrying various other sporadic mutations. Milner and Medcalf also looked at mutations at codon 273 (CGT->CCT and CGT->CTT) but did not investigate the particular recorded constitutional mutation at this site (CGT->CAG).

If we look at the overall frequency of total recorded mutations in those codons of p53 found constitutionally mutated (181, 242, 245, 248, 252, 258, 273, 282, 286, 307 and now 267), it is apparent that four of the sites are hypermutable (codons 245, 248, 273 and 282) where mutations account for approximately 25% of all those recorded in the p53 gene, codon 248 accounting for greater than 10% on its own (Caron de Fromentel & Soussi, 1991). Each of these sites contains a CpG dinucleotide (245 contains half a CpG dinucleotide) and 77% of the recorded mutations involve C->T changes at the CpG configuration. Although there is an under-representation of CpG dinucleotides in the vertebrate genome (Sved & Bird, 1990), it is known that 60–90% of them are methylated (Bird, 1986) and it is generally accepted that methylcytosine mutates at a high rate to thymine (Coulondre *et al.*, 1978). This type of spontaneous mutation, which occurs at 12 times the normal transition rate (Sved & Bird, 1990), is responsible for a high proportion of all p53 mutation found at these four hypermutable codons.

If we look only at the recorded constitutional mutations, half (10/19) occur at the four frequently mutated codons and nearly all of these (8/10) involve C->T mutations. Of the remaining nine (at codons 181, 242, 252, 258, 267, 286 and 307), two involve C->T changes at CpG dinucleotides. Three are at sites so far unique to constitutional mutations (181, 252) and the remaining six are at sites which are infrequently mutated (242, 258, 267, 286 and 307) and where the constitutional changes alone account for 53% of the recorded mutations.

In summary, more than half (10/19) of all constitutional p53 mutations appear to be spontaneous C->T changes at CpG dinucleotides and are frequently found at hypermutable sites (8/10). The codons of the remaining constitutional mutations are only infrequently mutated and have no consistent mutational pattern. (Five are C->T [G->A] in non-CpG dinucleotides, two are T->C [A->G], one is A->C [T->G], constituting seven transitions and one transversion, and one is loss of a single base.) The particular changes at the constitutional p53 mutations so far recorded are therefore consistent with the conclusion that endogenous spontaneous mutation could account for these events. The data are still too sparse to discuss 'hotspots' for constitutional mutations, but two codons (245 and 248) are responsible for eight of the 19 changes (42%). These are frequently mutated codons of the p53 gene in any case and are responsible for about 13% of all recorded mutations in the gene.

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p53 Mutations in Breast Cancer

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ABSTRACT

We have identified and analyzed 41 mutations in *p53* in sporadic breast tumors from 136 unselected breast cancer patients and estimate that approximately 40% of such tumors contain *p53* mutations. The frequency of G-T transversions and the incidence of guanosine mutations in the nontranscribed strand of the *p53* gene were found to be higher than expected, and we suggest, therefore, that exogenous carcinogens have an etiological role in sporadic breast cancers. Mutations were recorded in 44 codons of the *p53* gene, with no obvious mutational hot-spots, although mutations at codons 175, 194, 273, and 280 accounted for 25% of the changes. One germ-line mutation was found in 136 patients and so we conclude that constitutional mutation of *p53* may be an uncommon etiological factor in breast cancer.

INTRODUCTION

For female nonsmokers, cancer of the breast is the most important malignancy in Western society, and by the age of 75 approximately 10% of women in the United States will have developed the disease. There is evidence for a genetic contribution to the risk of developing breast cancer, as well as an association with modern affluence (diet and alcohol consumption). In addition, the influence of reproductive factors supports a hormonal role in the etiology of the disease (1, 2). Breast cancer is, however, a heterogeneous disease (3) and for this reason linkage studies to pinpoint a genetic locus or loci responsible for the inherited suceptibility have been beset with difficulties. Studies on LOH[3] in sporadic breast tumors have shown loss of genetic material at a number of loci including 1q, 3p, 6q, 16q, 17p, and 18q in >50% and at 1p, 7q, 8q, 9q, 11q, 13q, 15q, 17q, and 22q in >30% of tumors (4–17), suggesting the possible involvement of a number of tumor suppressor genes. In addition, several oncogenes (*myc*, *neu/HER-2*/c-*erb*B-2, and *int2*) have been implicated in the development of the disease (reviewed in Refs. 18 and 19). The etiology of breast cancer is, therefore, complicated both by disease heterogeneity and by the number and variety of genetic changes which appear to be important. However, one gene which is clearly involved in the development of both sporadic and some hereditary breast tumors is *p53* (20–34), a gene which in its unmutated form behaves as a tumor suppressor gene but which in at least several mutant forms acts as an oncogene (35).

Mutations in the *p53* gene are the most common genetic alterations in all human cancers (35, 36) and have been found at numerous sites in >100 of the 393 amino acids that comprise the protein (37, 38). Such a variety of mutations has permitted analyses for significant differences in the mutational spectra between different cancers (37) and has led to the postulation of etiological roles for particular mutagens, *e.g.*, the role of aflatoxin B1 and the hepatitis B virus in the G-T transversion of *p53* codon 249 in hepatocellular carcinomas from southern Africa and east Asia (39, 40). The nature of molecular change is often specific for a given mutagen, *e.g.*, UV light predominantly causes cyclobutane dimers (41) and *N*-methylnitrosourea produces G-A transitions (42). The nature of the mutations in *p53* may, therefore, point to the mutagen(s) involved. In skin cancers *p53* mutations at dipyrimidine sites (43) were indicative of the role of UV light, and in lung and esophageal cancers base pair changes in *p53* were characteristic of those produced by mutagens in tobacco and alcohol (44). The spectrum of mutations in radon-associated lung cancer in uranium miners is different from that usually seen in lung cancers and may reflect the genotoxic effects of radon (45). *p53* mutations in colon cancer are characterized by a very high incidence of C-T changes, mainly at CpG dinucleotides (37), although etiological reasons for this have not been advanced.

We have estimated the incidence of *p53* mutations in two series of sporadic breast tumors, based on mutation detection by the HOT detection technique (46). Using our own data on 137 tumors and published results of others, we discuss the overall incidence of *p53* mutations in sporadic breast tumors, the nature of these mutations, and their possible etiology. We have compared the spectrum of *p53* mutations in sporadic breast cancer with the germ-line mutations found in the Li-Fraumeni syndrome and with similar analyses in colon and other tumors.

MATERIALS AND METHODS

Tumor samples were collected from patients undergoing Patey mastectomy or wide local excision. All patients had presented with palpable breast lumps and had been referred by their general practitioners to the breast clinic in the Royal Infirmary of Edinburgh. Patients with T4 tumors or with distant metastases at presentation were excluded, because they were usually treated by chemotherapy in the first instance. Two sets of tumors resulted from serial admissions collected by two successive surgeons. All DNA preparations were made from whole blood or tumor tissue by standard techniques. PCR amplification, sequencing of PCR templates, and the HOT technique (HA and osmium tetroxide modifications) were carried out as previously described (21, 47). Scanning for mutations in exons 5 and 6 of the first 60-tumor set (mutations 1–8; Table 1) was carried out using HA modification only and labeled wild-type DNA; for mutations in exons 7, 8, and 9 of the 60-tumor set (mutations 9–16) both HA and OsO_4 modifications and labeled wild-type DNA were used. This was also done for exons 5 and 6 of the second 77-tumor set (mutations 17–32). HA and OsO_4 modifications and both wild-type and potential mutant labeled DNAs were used for exons 7–9 in the 77-tumor set (mutations 33–41); therefore, these fragments were scanned twice to ensure that all mutations were detected. The exact mutation in each instance was determined by direct sequencing of independently amplified PCR fragments. The leukocyte DNAs from patients with tumors carrying *p53* mutations were subsequently checked for germ-line mutations.

When estimating the frequency of *p53* mutations in breast tumors, a problem arises regarding the counting of multiple tumors from a single

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[3] The abbreviations used are: LOH, loss of heterozygosity; HOT, hydroxylamine and osmium tetroxide; HA, hydroxylamine; PCR, polymerase chain reaction.

Table 1 p53 *mutations found in 137 sporadic breast tumors, with LOH results for the same samples*

Mutation no.	Tumor no.	Codon	Nucleotide change	Protein change	LOH		
					BHp53	MCT35	YNZ22
1	8	164	AAG→CAG	Lys→Gln	U	U	L
2	21	136[a]	CAA→GAA	Gln→Glu	L	U	N
3	32	187[a]	GGT→TGT	Gly→Cys	L	U	L
4	34	175	CGC→CTC	Arg→Leu	L	U	U
5	36	201	TTG→TT-	Frameshift	U	N	L
6	43	179	CAT→GAT	His→Asp	L	L	L
7	51	152	CCG→TCG	Pro→Ser	N	L	U
8	85	157	GTC→TTC	Val→Phe		U	L
9	4	250	CCC→GCC	Pro→Ala	N	L	N
10	8	265	CTG→CCG	Leu→Pro	U	U	L
11	9	281	GAC→GGC	Asp→Gly	N	L	L
12	20	307	GCA→ACA	Ala→Thr	L	L	L
13	33	283	CGC→CCC	Arg→Pro	U		N
14	47	280	AGA→GGA	Arg→Gly	N	U	L
15	57	242	TGC→TTC	Cys→Phe	L	L	L
16	80	267	CGG→CAG	Arg→Gln	N	U	L
17	8	208	GAC→GTC	Asp→Val	—	—	—
18	10	194	CTT→CGT	Leu→Arg	—	—	—
19	11	140	GAC→G-C	Frameshift	—	—	—
20	18	179	CAT→TAT	His→Tyr	L	U	L
21	21	192	CAG→TAG	Gln→Stop	L	L	L
22	25	186	GAT→TAT	Asp→Tyr	—	—	—
23	34	182	TGC→TGA	Cys→Stop	U	L	N
24	36	178	CAC→CCC	His→Pro	U	U	L
25	57	175	CGC→CAC	Arg→His	L	L	
26	64	213	CGA→TGA	Arg→Stop	N	U	L
27	67	163	TAC→AAC	Tyr→Asn	U	U	L
28	71	141	TGC→TAC	Cys→Tyr	—	—	—
29	72	194	CTT→CGT	Leu→Arg	L	U	
30	95	213	CGA→TGA	Arg→Stop	U	U	L
31	113	194	CTT→CGT	Leu→Arg	—	—	—
32	114	175	CGC→CAC	Arg→His	—	—	—
33	8	285	GAG→AAG	Glu→Lys	—	—	—
34	12	276	GCC→CCC	Ala→Pro	—	—	—
35	61	273	CGT→CAT	Arg→Leu	—	—	—
36	84	273	CGT→CTT	Arg→Leu	—	—	—
37	88	237	ATG→ATT	Met→Ile	—	—	—
38	92	255	ATC→- - -	Ile→Del	U	—	—
39	98	239	AAC→GAC	Asn→Asp	N	U	—
40	101	245	GGC→GAC	Gly→Asn	L	U	L
41	110	282	CGG→CTG	Arg→Leu	—	—	—

[a] Thompson *et al.* (32) give incorrect sequence changes for mutations at codons 136 and 187. In addition, no mutations were found in tumors 65 and 81 (codons 194 and 67) of their list. L, loss; N, no loss; U, uninformative; —, not done.

individual. If multiple tumors unequivocally represented multiple primaries, then, for the purposes of estimating mutation frequency, each tumor would be considered independently. But differentiating between multiple independent primaries and multiple deposits of a single primary is difficult. We consequently made the following decision: six of the 136 patients had multiple tumors (total of 14 tumors); in five individuals the *p53* mutation status of each of the multiple tumors was identical and these were, therefore, counted as five tumors in total; in one individual with two tumors, one tumor contained mutant *p53* and one did not, and these were counted as two separate tumors.

RESULTS AND DISCUSSION

Incidence of *p53* Mutations in Sporadic Breast Cancer. We have found 41 mutations in sporadic tumors from 136 unselected breast cancer patients (137 tumors, as detailed in "Materials and Methods"). The precise codon alterations are listed in Table 1 and sequence data on a number of mutations are shown in Fig. 1. Two tumors contained two independent mutations. In a separate study,[4] it was determined that the HOT technique as used (see "Materials and Methods") detects 90% of all mutations. For the purpose of estimating mutation frequency, we consider only the second 77-tumor set, in which 25 tumors (31%) contained mutations in exons 5–9 of the *p53* gene. An allowance for the incomplete detection of mutation suggests a probable frequency closer to 36%.

Our study does not take into account the total number of possible mutations in the entire *p53* gene. In collated published data regarding alterations to *p53* in a wide variety of cancers (38), 6% of all mutations (22 of 368) were recorded outside exons 5–9, in exons 1–4, 10, and 11 and in introns 3, 4, 5, 6, 7, and 9, despite a heavy bias in the literature against analyzing these regions. The 6% of mutations found outside exons 5–9 could, therefore, be a notable underestimate. Although few such studies have been undertaken, the sequencing of several entire *p53* cDNA copies in a variety of tumors and tumor cell lines did not reveal mutations which lie outside this region (20, 48–50). In addition, although we have included introns 5, 7, and 8 in the PCR fragments of our survey, we have found no splicing mutations in 137 tumors. If approximately 10% of all *p53* mutations lie outside exons 5–9, then the incidence of mutation in the entire *p53* gene in sporadic breast tumors must be on the order of 40%. This estimate is in accord with, and extends, published findings on smaller series using nonimmunohistochemical methods (single-strand conformation polymorphism, HOT, constant denaturing gel electrophoresis, etc.) for mutation detection (see Table 2).

Published estimates of mutations detected by immunohistochemical techniques suggest that on the order of 60% of sporadic breast tumors have mutations in the *p53* gene (23, 27,

[4] Condie, A., Eeles, R., Børreseu, A-L., Coles, C., Cooper, C., and Prosser, J. Detection of point mutations in the *p53* gene: comparison of SSCP, CDGE, and HOT, submitted for publication.

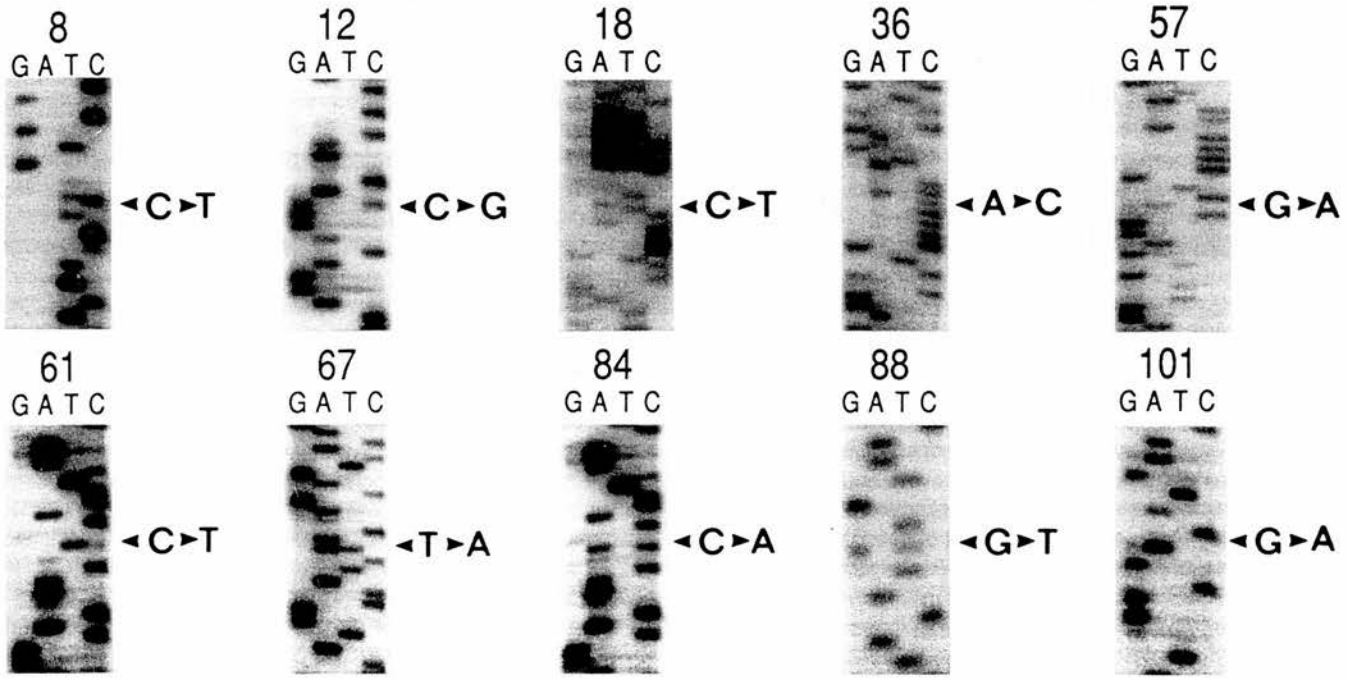

Fig. 1. Sequencing data for 10 tumors in which the relative contributions of normal and wild-type alleles can be assessed. For example, in tumor 8 the amount of wild-type DNA is greater than that of mutant DNA, in tumor 61 the amount of mutant DNA is greater than that of wild-type DNA, and in tumor 57 only mutant DNA is present.

Table 2 *Proportion of sporadic breast tumors with mutations in the* p53 *gene (nonimmunohistochemical methods of detection)*

Using DNA-based methods of mutation detection [constant denaturing gel electrophoresis (CDGE), (single-strand conformational polymorphism (SSCP), HOT, direct sequencing], various regions of the *p53* gene in breast tumors have been examined. The incidence of mutations has been compared in exons 5–8. In order to compare these estimates with those achieved by antibody staining, we have estimated mutation incidence in the entire *p53* gene (see text).

Method	Tumors examined	Tumors with mutation	Exons studied	Reported mutations (%)	Estimated mutations in exons 5–8 (%)	Estimated mutations in all exons (%)	Ref.
HOT	77	25	5–9	32	36[a]	40	This paper
HOT	60	16	5–9	27	33[b]	37	21, 32
CDGE	32	10	5, 7, 8	31	34[c]	38	29
SSCP	24	9	5–8	38		42	30
SSCP	59	10	5–9	17		19	31
PCR	11	4	5–9	36		36	28
RNase protection	26	3	Entire gene	12			30
PCR	59	21	2–11	36			31

[a] If 25 represents 90% of mutations present, then 28 mutations would be the probable actual incidence in these 77 tumors (36%) (see text).

[b] Prosser *et al.* (21) used the HOT technique with HA modification and wild-type labeled DNA. This theoretically detects 50% of all possible mutations but in sporadic breast tumors, where 74% of mutations occur in GC base pairs, approximately 70% are detected. Therefore, the 8 mutations found in exons 5 and 6 would represent 11 probable mutations. The HOT technique with wild-type DNA labeled using both HA and OsO_4 modifications detects 90% of mutations.[4] Consequently, the 8 mutations found in exons 7, 8, and 9 (32) would represent 9 probable mutations, giving a total of 20 probable actual mutations in the 60-tumor set (33%).

[c] Exons 5, 7, and 8 were studied. Mutations in exon 6 account for 13% of *p53* mutations recorded between exons 5 and 8 in all cancers surveyed (38). This estimate has, therefore, been adjusted to include potential exon 6 mutations.

51, 52). Immunohistochemical staining uses *p53*-specific antibodies which recognize various epitopes on the *p53* protein. The increased stability of many mutant *p53* proteins permits their detection in tissue sections with antibodies such as pAb421, pAb1801, and polyclonal CM-1. Antibody pAb240 specifically recognizes an epitope characteristic of many mutant proteins (53).

There is clearly a serious discrepancy in the estimates of mutation based on the two sorts of technique, one an analysis at the DNA level (~40%) and the other at the protein level (~60%). This might suggest that sampling bias in the DNA studies may be greater than estimated and that >10% of mutations may occur outside exons 5–9. On the other hand, the assumption that detection of expression of p53 protein means overexpression of mutant as opposed to wild-type protein may be open to question. Indeed, some tumorigenic cell lines overexpress wild-type *p53* (54), and it is known that there are differences in p53 protein levels due to factors affecting differentiation (55) and cell cycle progression (54). Moreover, in four undifferentiated neuroblastoma-derived cell lines high-level expression of a stable, apparently wild-type, protein was found (56). It is of course possible that a gene outside the *p53* locus is involved in the increased half-life of some proteins. Milner and Watson (57) observed that the wild-type murine p53 protein could be expressed in a form immunologically similar to a mutant protein, within 1 h after addition of fresh growth medium. If their observations hold true in human material, it is possible that not all the p53 which stains with mutant-specific antibody is mutant. Interestingly, the temperature-sensitive p53 mutant (Val-135) fluctuates between normal and mutant protein conformation depending on the growth temperature (58). Overall, there would appear to be good reason to question the

assumption that elevated expression of p53 is synonymous with expression of mutant p53.

Notwithstanding the qualifications given above, in studies of 24 positively staining breast tumors and cell lines known to overexpress mutant p53, 22 were shown to possess mutations in the gene (22, 24, 25, 27, 29), and similar findings have been reported for tumors and cell lines of lung, colon, ovary, and esophagus (44, 59–62). In fact, there are a number of reasons to suppose that some mutant *p53* genes would be undetected by antibody staining, *i.e.*, those with stop codons truncating the gene before the recognized epitope, those with deletions of both alleles, and those with protein degraded by the human papillomavirus E6 protein. Some investigators have, indeed, found mutations in negatively staining material (29, 44, 60). At present, therefore, in the absence of direct comparison within a large series of mutations detected both at the DNA level and at the protein level, it is unclear whether DNA studies underdetect mutations or antibody-staining techniques overdetect them.

Relationship between *p53* Mutation and LOH on Chromosome 17p. The *p53* gene is located at 17p13, and data on *p53* mutation and LOH for this region can indicate a temporal order for the two events in tumor development. Our results show that, of 20 tumors which are both mutant at the *p53* gene and informative for markers at BHp53 or MCT35 (loci near *p53*), 75% show LOH (Table 1) and may conform to the sequence of events of mutation in *p53* followed by loss of the normal allele on the partner chromosome. This value is similar to those found in other breast cancer studies also using markers near the *p53* gene (24, 29, 30). Evidence for two independent loci showing significant LOH on the short arm of chromosome 17, one at 17p13.1, the site of *p53*, and one more telomeric to this at 17p13.3, has been found in a number of cancers including breast (8, 11), liver (63), and kidney (64). Unless the pattern of genetic loss on 17p has been shown to involve only one locus, as is the case with colon cancer (48), LOH over the *p53* gene cannot be assumed by loss at a more telomeric site (*e.g.*, that defined by the probe YNZ22).

Several investigators have directly addressed the question of the presence or absence of a wild-type allele in tumors containing a mutation in *p53*, using sequence data or single-strand conformation polymorphism autoradiographs. Since whole tumor DNAs from our breast tumor series were PCR amplified and sequenced with no consideration for the relative amounts of tumor and normal tissue in each of the samples, in many instances it was difficult to determine whether the mutations in *p53* were accompanied by loss of the wild-type *p53* allele. In our 77-tumor series, sequencing data showed that the mutant allele was at least twice the intensity of the wild-type allele in 11 of the 25 mutations, evidence that, in the tumor tissue, the mutant allele was accompanied by loss of the normal allele. In the remaining 14 mutations, however, the wild-type allele was either equal to or greater than the mutant allele in intensity. In the literature, a high proportion of homozygous or hemizygous mutations in breast tumors and cell lines has been found (24, 28, 30, 31), and a similar high proportion of homo/hemizygosity pertains to tumors of the ovary, brain, liver, lung, uterus, and gut, as well as leukemia (39, 65–71). In the two tumors containing two independent *p53* mutations, no LOH data are available; neither is it known if the mutations are on separate alleles.

While in a proportion of sporadic breast tumors, as in colon cancer (20, 48, 72), *p53* mutation may occur before loss of the wild-type allele, there are a substantial number in which loss is not accompanied by mutation. Of 34 tumors in our series with LOH near the *p53* gene 56% apparently have only normal *p53* DNA, suggesting that in many tumors loss of the normal wild-type allele may occur without concomitant *p53* mutation. Other studies with similar results (20, 24, 30, 48, 70) suggest several possible explanations for this: that loss on 17p may be a random or nonselective loss, that the screening technique used may not have detected the corresponding *p53* mutation, that *p53* mutation may occur outside the region screened, that there may be a dosage effect such that 50% dosage of *p53* confers a selective growth advantage to the cell, or, finally, that the allele loss may involve a second tumor suppressor gene.

Mutation of one copy of *p53* and loss of the wild-type allele conform to Knudson's two-hit hypothesis of tumor suppressor genes, and our results support the role of *p53* as a tumor suppressor gene in a number of breast tumors. Nevertheless, it should be remarked that we, and others (25, 30), have found evidence for a proportion of tumors with *p53* mutation and no concomitant loss of the remaining wild-type allele (five in this study with no LOH of adjacent 17p markers). It is known that certain mutant forms of *p53* behave as oncogenes (73–75), and it would be interesting to address the question of whether strongly oncogenic mutations require no loss of the wild-type *p53* allele in order to effect a growth advantage, while mutations which are either weakly oncogenic or not oncogenic (simply loss of function) need to be associated with loss of the normal allele. The relative oncogenicity of only a few mutant *p53* genes has been determined. Hinds *et al.* (73) and Halevy *et al.* (74) have compared different mutant *p53* genes for their ability to transform primary embryo rat cells in culture. Milner and Medcalf (75) have compared mutant *p53* genes for their ability to drive wild-type *p53* into the mutant conformation. From these studies, a number of mutations have been catergorized as strongly oncogenic (Phe-132, Val-135, Ser-151, His-175, Ile-247, and Pro-273) or weakly oncogenic (Trp-248, Cys-270, His-273, and Gly-281). Only two samples from our breast tumor series contain mutations of tested oncogenicity. Tumors 25 and 32 (Table 1) both have the 'strong' His-175 mutation and so might be expected to have retained the wild-type *p53* allele, but tumor 25 shows LOH at BHp53 and MCT35.1 and sequencing data show complete and partial loss of the wild-type allele in tumors 25 and 32, respectively.

Analysis of the *p53* Mutational Spectrum in Sporadic Breast Tumors. A total of 84 mutations in sporadic breast tumors from our series and from the literature are listed in Table 3. When the specific types of mutations are analyzed (Table 4), there are some notable observations. There is an excess of point mutations at G/C base pairs (75%). (The overall content of GC in the *p53* gene is 56%.[5]) It is possible that 50% (42 of 84) of all breast tumor mutations are point mutations occurring at guanosines in the noncoding strand. This bias is seen in GC-CG, GC-AT, and GC-TA mutation events, although it is most pronounced for GC-TA transversions (14 of 16 or 88%). Eleven of 14 GC-AT transitions at CpG dinucleotides may be more realistically viewed as C-T alterations in the coding strand, but the remaining 31 of 84 mutations (37%) which could have occurred at guanosines in the noncoding strand are in excess of expectation. This is significant in view of the fact that the nucleoside guanosine is a preferential target for most chemical

[5] P. M. Chumakov, EMBL Accession Number is X54156 for HSP53G.

Table 3 *Codon number and specific alteration for breast tumor mutations (mutations from this paper and from the literature)*

Single nucleotide substitutions

Codon number	Codon change	Nucleotide change	CpG dinucleotide	Source	Ref.
128	CCT→CCG	T→G		Tumor	29
128	CCT→TCT	C→T	Non	Tumor	29
132	AAG→CAG	A→C		Cell line	22
134	TTT→CTT	T→C		Tumor	29
136	CAA→GAA	C→G		Tumor	21
141	TGC→TAC	G→A	Non	Tumor	This paper
151	CCC→TCC	C→T	Non	Tumor	26
152	CCG→TCG	C→T	Non	Tumor	21
157	GTC→TTC	G→T		Tumor	21
157	GTC→TTC	G→T		Cell line	28
163	TAC→AAC	T→A		Tumor	This paper
163	TAC→TGC	A→G		Tumor	24
164	AAG→CAG	A→C		Tumor	21
175	CGC→CAC	G→A	CpG	Tumor	This paper
175	CGC→CAC	G→A	CpG	Tumor	This paper
175	CGC→CAC	G→A	CpG	Tumor	27
175	CGC→CAC	G→A	CpG	Tumor	29
175	CGC→CTC	G→T		Tumor	21
175	CGC→CAC	G→A	CpG	Cell line	28
178	CAC→CCC	A→C		Tumor	This paper
179	CAT→TAT	C→T	Non	Tumor	This paper
179	CAT→GAT	C→G		Tumor	21
182	TGC→TGA	C→A		Tumor	This paper
186	GAT→TAT	G→T		Tumor	This paper
187	GGT→TGT	G→T		Tumor	21
192	CAG→TAG	C→T	Non	Tumor	This paper
193	CAT→CCT	A→C		Tumor	*a*
194	CTT→CGT	T→G		Tumor	This paper
194	CTT→CGT	T→G		Tumor	This paper
194	CTT→CGT	T→G		Tumor	This paper
194	CTT→TTT	C→T	Non	Cell line	20
196	CGA→CCA	G→C		Tumor	*a*
208	GAC→GTC	A→T		Tumor	This paper
213	CGA→TGA	C→T	CpG	Tumor	This paper
213	CGA→TGA	C→T	CpG	Tumor	This paper
234	TAC→TAA	C→A		Cell line	31
237	ATG→ATT	G→T		Tumor	This paper
237	ATG→AAG	T→A		Tumor	29
237	ATG→ATA	G→A	Non	Tumor	24
238	TGT→TTT	G→T		Tumor	30
238	TGT→TTT	G→T		Tumor	29
239	AAC→GAC	A→G		Tumor	This paper
242	TGC→TTC	G→T		Tumor	32
245	GGC→GAC	G→A	Non	Tumor	24
245	GGC→GTC	G→T		Tumor	29
245	GGC→GAC	G→A	Non	Tumor	This paper
248	CGG→TGG	C→T	CpG	Tumor	29
248	CGG→CAG	G→A	CpG	Tumor	24
248	CGC→CAG	G→A	CpG	Tumor	25
249	AGG→AGC	G→C		Cell line	22
250	CCC→GCC	C→G		Tumor	32
254	ATC→AAC	T→A		Tumor	25
265	CTG→CCG	T→C		Tumor	32
266	GGA→GTA	G→T		Tumor	25
267	CGG→CAG	G→A	CpG	Tumor	32
273	CGT→CAT	G→A	CpG	Cell line	20
273	CGT→CTT	G→T		Tumor	This paper
273	CGT→CAT	G→A	CpG	Tumor	This paper
273	CGT→CAT	G→A	CpG	Tumor	29
276	GCC→CCC	G→C		Tumor	This paper
278	CCT→GCT	G→C		Tumor	24
280	AGA→ACA	G→C		Tumor	30
280	AGA→ACA	G→C		Tumor	28
280	AGA→GGA	A→G		Tumor	32
280	AGA→AAA	G→A	Non	Cell line	22
281	GAC→GGC	A→G		Tumor	32
281	GAC→GGC	A→G		Tumor	29
282	CGG→CTG	G→T		Tumor	This paper
282	CGG→CCG	G→C		Tumor	27
282	CGG→CTG	G→T		Tumor	24
283	CGC→CCC	G→C		Tumor	32
285	GAG→AAG	G→A	Non	Tumor	This paper
285	GAG→AAG	G→A	Non	Tumor	30
285	GAG→AAG	G→A	Non	Cell line	22
307	GCA→ACA	G→A	Non	Tumor	32

Table 3—*Continued*

Deletions

Codon	Codon change	Deletion	Source	Ref.
140	GAC→G–C	AT base pair	Tumor	This paper
167	CAG→CA–	GC base pair	Tumor	27
172	GTT→GT	TA base pair	Tumor	29
175–180	Deletion of 6 codons	18 base pairs	Tumor	24
201	TTG→TT–	GC base pair	Tumor	21
235–239	Frameshift deletion	14 base pairs	Tumor	28
255	ATC deletion	3 base pairs	Tumor	This paper
329	ACC→AC–	CG base pair	Tumor	28
Exon 11	Deletion of 30 base pairs	30 base pairs	Tumor	30

[a] P. Devilee, unpublished observations.

carcinogens (76) and that the nontranscribed strand is more commonly the site of damage (possibly because of a bias in the rate of DNA strand repair such that the coding strand is preferentially repaired over the noncoding strand) (77).

Two categories of mutation are, therefore, noticeably prevalent in sporadic breast tumors: CG-TA transitions and GC-TA transversions. The increased incidence of CG-TA changes occurs both at CpG dinucleotides (19%) and at cytidines and guanosines not contained in this sequence conformation (20%). Although CG-TA changes at CpG dinucleotides are common, they are not as frequent as in colon cancer, where they account for 67% of the total changes (37). With GC-TA transversions, it is apparent that they are more frequent in sporadic breast cancer than expected. Hollstein *et al.* (37) found that GC-TA changes occur at high frequency in lung cancer (non-small cell lung cancer, 57%), liver cancer (74%), and esophageal cancers (24%), where a number of specific mutagenic factors are believed to be important. Aflatoxin B1 is a potent liver carcinogen which induces G-T transversions, and carcinogens in tobacco (for example, benzo[*a*]pyrene) are also known to elicit G-T transversions. However, in other solid tumors (colon, bladder, ovary, sarcomas, and brain) GC-TA transversions constitute a small proportion of all mutations (5–13%). Of 75 point mutations in breast tumors in our series, 16 (21%) are GC-TA transversions. The increased frequency of GC-TA transversions and the high incidence of mutation of guanosines in the noncoding strand of the gene might, therefore, imply that external carcinogens have a role in the development of sporadic breast tumors.

The germ-line mutations to *p53* found in the Li-Fraumeni familial cancer syndrome (which includes breast tumor) have a mutational spectrum with a preponderance of CG-TA transitions and few GC-TA transversions (Table 4). Forty-four % of the germ-line mutations are at CpG dinucleotides, a change that is frequent in mammalian genomes (78) and may be attributed to spontaneous deamination of cytosine. It, therefore, appears that different factors predispose cells to the somatic *p53* mutations found in a large proportion of sporadic breast tumors and to the germ-line *p53* mutations which occur as part of the Li-Fraumeni inherited cancer syndrome.

When the mutational spectra between breast cancer and colon cancer are compared, there are obvious differences. A high proportion (67%) of all *p53* mutations in colon cancer are CG-TA transitions at CpG dinucleotides. There are no recorded GC-TA transversions (37, 38) and three codons are indisputably hot-spots for mutation: codons 175, 248, and 273

Table 4 *Frequency of specific mutations in breast tumors, in Li-Fraumeni patients, and in cancers other than breast*

	GC-CG	GC-TA	GC-AT (CpG, non)	AT-GC	AT-CG	AT-TA	Total
Published breast mutations except those from this laboratory (20, 22, 24–31)[a]	6	7	16 (8, 8)	3	3	2	37
Breast mutations from this laboratory (this paper; Refs. 21 and 32)	5	9	13 (6, 7)	4	5	2	38
Total breast tumor mutations	11	16	29 (14, 15)	7	8	4	75
Percentage of specific mutations in breast tumors	15	21	39 (19, 20)	9	11	5	100
Germ-line *p53* mutations (mainly Li-Fraumeni)	0	2	15 (10, 5)	3	1	0	21
Percentage of specific germ-line mutations	0	10	71 (47, 24)	14	5	0	100
Percentage of specific *p53* mutations in all cancers other than breast (38)	9	21	48 (36, 12)	11	5	5	100
Percentage of specific *p53* mutations in all cancers other than breast, liver, and lung (38)	8	11	56 (42, 14)	13	6	5	100

[a] P. Devilee, unpublished observations.

(accounting for >50% of all the alterations). In contrast, in breast cancer the picture is very different; of the 75 point mutations analyzed, only 19% occur at CpG dinucleotides, 21% are GC-TA transversions, and only 24% of breast cancer mutations occur at four codons: 175 (six), 194 (four), 273 (four), and 280 (four). Overall, CG-TA transitions at CpG are less frequent, GC-TA transversions are more frequent, and there are not such pronounced hot-spots for mutation as found in colon cancer.

Analysis of the Particular Mutations and Codons Involved. Data from a number of studies show that the most frequent type of change occurring in the *p53* gene is a single-base missense substitution which alters a single amino acid in the protein (*e.g.*, Refs. 20, 37, and 38), and this is true in breast tumors also, where 89% (75 of 84) are single-nucleotide substitutions (84% result in amino acid substitutions and 5% in stop codons). The remaining 11% (9 of 84) are deletions. The locations of all point mutations in sporadic breast cancer published to date (Refs. 20–32, 37, and 38 and this paper) have been in conserved codons of the gene; 68% are in conserved domains II to V, 8% in domain II, 12% in domain III, 23% in domain IV, and 25% in domain V. Of the remaining mutations, 17 of 24 occur at codons conserved from *Xenopus* to mammals and the rest are found in codons conserved among mammals. A number of neutral mutations and polymorphisms have been reported for *p53* in the literature. In 76 patients we have found two samples with the CGA-CGG neutral mutation constitutionally present at codon 213 (79, 80).

One of the 41 mutations in 136 patients was found to be a constitutional change, CGG-CAG at codon 267, giving an incidence of constitutional mutations in *p53* in sporadic breast cancer of 0.7% (81). The patient carrying this constitutional mutation was subsequently found to have come from a cancer-prone family, although not a Li-Fraumeni family. We have reanalyzed the five breast cancer-prone families previously reported (47) and confirm our inability to find constitutional *p53* mutations in these individuals. In our 77-tumor set we found no relationship between *p53* mutation and tumor size, disease stage, or menopausal status. This was also true for the 60-tumor set (32).

Conclusions. (*a*) From our study of 136 breast cancer patients we estimate that 40% of sporadic breast tumors carry mutations in the *p53* gene. A review of the data on incidence of *p53* mutations in sporadic breast cancer shows that the estimates of mutation frequency are significantly different depending upon whether the measurements are made by DNA analysis (~40%) or by immunohistochemical methods (~60%). There are theoretical reasons for both techniques to underestimate the actual incidence, but there is good evidence to suggest that immunohistochemical staining may well result in an overestimation. Until a body of material selected on the basis of immunohistochemical screening is extensively analyzed by non-immunohistochemical methods and/or sequencing, it will be difficult to account for the observed differences.

(*b*) In sporadic breast tumors, the increased frequency of GC-TA transversions, together with a very high incidence of guanosine mutations in the nontranscribed strand of the *p53* gene, leads to the conclusion that exogenous carcinogens may have an etiological role in these tumors. Information was not available on what proportion of the breast cancer patients were smokers and whether this was relevant in accounting for some G-T transversions. Carcinogenic hormones are generally inefficient in the production of point mutations at the gene level (82), but increased estrogen levels are known to promote cell growth and may indirectly increase the incidence of mutation.

(*c*) Germ-line *p53* mutations in the Li-Fraumeni family cancer syndrome (which includes breast cancer) are mainly CG-TA transitions at CpG dinucleotides. These may be naturally occurring endogenous events.

(*d*) Somatic mutations in breast tumors have been found in 44 codons of the *p53* gene between exons 5 and 9. A preponderance of mutations was found at codons 175, 194, 273, and 280, but no particular mutational hot-spot was identified.

(*e*) Analysis of the data for LOH and mutation in *p53* in sporadic breast tumors suggests that in a proportion of tumors mutation may occur before allele loss but this sequence of events is not followed in a substantial number of tumors.

(*f*) In breast cancer patients an autosomal dominant mode of inheritance accounts for approximately 5–10% of cases (83). Based on our finding of one germ-line mutation in sporadic breast tumors from 136 patients (81) and on our finding of no apparent *p53* mutation in five families with breast cancer (47), we conclude that mutation in *p53* is a rare etiological factor in hereditary breast cancer.

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